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Plant Oils as Fuels

Present State of Science and Future Developments

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N. MARTINI J. SCHELL

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Present State of Science and Future Developments

Proceedings of the Symposium
held in Potsdam, Germany
February 16–18, 1997

With 98 Figures and 79 Tables

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Foreword

Among renewable energy resources, Biodiesel fuel made from rapeseed is of special importance in Europe. The discussion on Biodiesel is, however, somewhat controversial. Economical, technological, ecological and toxicological arguments have been advanced implying that, at present, Biodiesel is at best just a "niche" product that can only compete with traditional fossil diesel fuel because of significant tax incentives. Indeed, the economic disadvantages of producing Biodiesel are not compensated by the technological, ecological and possibly toxicological advantages. Given the present state of knowledge in these very different areas, the decisive question to be asked is whether the competitiveness, and thus marketability, of Biodiesel can be enhanced by biotechnological manipulations of the rape plant. Apart from purely economical aspects, such as increasing crop yields, decreasing production costs and increasing technological quality, attention must be focussed especially on reducing potential dangers to man and the environment.

The symposium on Plant Oils as Fuels was intended as a forum for economists, engineers, ecologists and toxicologists to present and exchange their ideas for improving the product Biodiesel and to discuss these views with expert plant bioscientists. The challenge for the latter was and will be to determine whether biotechnological methods can be applied safely to introduce new properties into the rape plant, an example for a number of other plants also suitable for production of renewable energy. The properties concerned are those which will improve the product Biodiesel fuel economically, technologically and especially toxicologically such that it will then possess those characteristics identified as the most desirable by the scientists. It was, therefore, not the goal of the symposium to evaluate Biodiesel as a form of energy under the present conditions, but rather to find an answer to the question of the chances Biodiesel will have in the market of the future.

The VERUM-Foundation - Foundation for Behaviour and Environment, Munich, - promotes research into the interrelationships between man and his environment. As a non-profit-making scientific organization the Foundation supports important scientific work within this research area. Plant oils as renewable energy sources deserve special attention because of the implications for the wellbeing of future generations of mankind. Society should have the opportunity to form opinions on matters of relevance based on the results of scientific work and free from the preconceived notions of ideologies or vested interests. Knowledge-based opinions should be the presupposition in decision making, both at the individual and political level.

Munich, February 1998

Prof. Dr. med. Dr. h.c. Klaus Thurau
Chairman of the Board

Prof. Dr.med. Franz Adlkofer
Executive Director

Acknowledgement

The Symposium on Plant Oils as Fuels - Present State of Science and Future Developments - took place on February 16-18, 1997 at the beautiful ambiente of Schloß Cecilienhof in Potsdam, Germany. The atmosphere of such a historic place greatly stimulated the scientific exchange and discussions of the participating experts.

With this in mind, the editors would like to give credit to all who helped that this Symposium was very successful, in particular:

We gratefully acknowledge the generous support of VERUM foundation, Munich, both for the Symposium and for the Proceedings.

The speakers are gratefully acknowledged for their time and effort preparing their lectures and manuscripts. Without their expertise and dedication the Symposium could not have taken place.

We wish to thank Dr. Günter Oberdörster and Dr. Peter Walzer, who invited experts of their fields to participate in this interdisciplinary symposium and supervised the anonymous peer-review process of the manuscripts of their sessions prior to acceptance for these Proceedings. Our thanks to John M. Davis, PhD, for language editing of the recorded discussion sections.

We are grateful to Ms. Ursula Kerscher, Ms. Eva Kath, Ms. Regina Droll and Dr. Gisela Henze for their attentive assistance that everyone was well taken care of during the Symposium and that everything was extremely well organized.

The Proceedings could have failed without major contribution of Dr. Jutta Weinand whose most excellent and dedicated editorial efforts in preparing the camera-ready manuscript are gratefully acknowledged.

N. Martini and J. Schell
February 1998
Max-Planck-Institute for Breeding Research
Cologne, Germany

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Introduction
The Biodiesel Market Today and its Future Potential
(*Chairman: Jeff Schell*)

The Biodiesel Market Today and its Future Potential

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Abstract

Having been mentioned already by Rudolf Diesel plant oils represent not a revolutionary alternative fuel compared to fossil sources, but only by the force of the oil supply shocks in the 70s a consequent development of Biodiesel was triggered. This paper gives a review of the political background, the historical development since the beginnings in European countries, the volumes produced by various process technologies and the main raw materials used. It highlights the development of Biodiesel fuel standards as a needed measure for quality assurance, the various engine performance properties and the fuel's environmental advantages. The different marketing strategies applied are shortly described as well as the key factors of micro- and macroeconomic considerations.

As an introduction for the other papers the future potential for Biodiesel is pointed out, based on available agricultural land in the enlarged EU and based on a broad variety of suitable and improved oilseeds with modified oil profiles. New energy policies, which strongly require the development of renewable fuels for the transport sector, new environmental guidelines for special fuels in endangered sectors and new recommendations for employment creation in rural community areas are mentioned.

Triggers for Biodiesel and the political background

The basic principles of using plant oils as a fuel for diesel-engines were already known and applied by Rudolf Diesel himself and mentioned in his patent application in 1912. A new impulse however was given by the crisis in supply of mineral oil as the major source for energy in the 70s and again by the Gulf war in 1991.

Being highly dependent on huge imports of fossil oil - as a finite energy source anyway - the European Union has to face today again an increasing risk in security of energy supply for the transport sector caused by the following issues as emphasised by the International Energy Agency (IEA):

- the production-demand gap of fossil oil is declining world-wide,

- North Sea oil will be finished by the year 2010 latest, and
- the energy demand of the non-OECD world is growing dramatically (e.g. China).

According to the IEA there will be a need for *all* alternative fuels for the transport sector, and Biodiesel will be one of them [1].

As far as environmental damage is concerned, the transport sector has a clear responsibility. Within the last 10 years its part in global warming potential has increased from less than 20 % to more than 25 %; it is now bigger than those of the domestic and industrial sector, while its contribution to acid pollution constitutes 75 % of total emissions of this pollution type. As one reaction the European Commission has developed a fuel quality directive asking for new environmental fuel specifications aiming at the reduction of exhaust and evaporative emissions [2].

Substantial and costly overproduction of agricultural crops for food has led to a reformed Common Agricultural Policy in recent years introducing a set-aside percentage for food-crop production but allowing to produce crops for non-food purposes on those fields. Non-food rapeseed grown on set-aside fields was by far the major source for Biodiesel in the past years. But the initial percentage for set-aside of 15 % declined step by step over the years to 5 % today, putting the young Biodiesel-industry at a substantial risk of raw material shortage.

The future agricultural policy however will have to take into consideration, that with the enlargement of the European Union by the Countries of Central Europe (CCE) tremendous opportunities for Biodiesel production are opening up, as

- those countries have presently double the acreage per citizen compared to the EU-15,
- they will represent 55 % of the total agricultural land within the new EU-25,
- there is an enormous unexploited potential in agro-productivity.

This challenge of integration can lead to a significant impulse in production of renewable raw materials in general and of Biodiesel in specific.

Review of milestones in the Biodiesel development

It was around 1982 when the Institutes for Agricultural Engineering in Braunschweig/Germany and Wieselburg/Austria tested Rapeseedoil-Methyl-Ester (RME) in Diesel engines for the first time. Already in 1985 a small pilot plant at the agricultural highschool in Silberberg/Austria tested the production of RME with a new technology (ambient pressure and temperature) and in 1990 the first farmers' co-operative in Asperhofen/Austria started commercial production of Biodiesel derived from rapeseed as well as sunflower. In the same year the completion of a large fleet tests of all major tractor brands was completed at Wieselburg and led to engine warranties by most of the tractor producers as e.g. John Deere, Ford, Massey-Ferguson, Mercedes, Same a.o., as a big step forward towards a successful market introduction of Biodiesel.

Another important goal was achieved in 1991 with the issue of the first fuel standard ON C 1190 for Biodiesel by the Austrian Standardisation Institute

assuring engine producers and end users a high quality of the fuel. Detailed tests on product properties such as engine performance, emission reductions, biodegradability and toxicity were now conducted in more and more countries, leading to a complex understanding of the fuel and its properties, while economical investigations were going on as well.

The year 1996 was marked by the start-up of large industrial scale plants in Rouen/France and in Leer/Germany; and by the milestone of overall warranty for all models of Volkswagen and Audi as trailblazers in the personal car sector. In the same year the foundation of the European Biodiesel Board as a professional organisation of all major Biodiesel producers took place, indicating the further growth of a young industry.

Biodiesel production world-wide in now estimated according to the following table with France, Italy and Germany as the leading countries up till now:

Table 1. Capacities and production development

Biodiesel estimated/000 mt:	1996 capacities	1996 production	1997 estimate	projects
Austria	38	17	22	30
Belgium	200	20	20	0
France	310	227	250	100
Germany	291	63	83	30
Great Britain	2	2	2	0
Italy	199	141	109	0
Sweden	11	3	8	10
DK, IRL	1	1	1	30
EU - 15	1.052	474	495	200
Czechia	63	22	45	0
Rest of Europe	10	8	10	5
U.S.A.	38	5	8	150
Canada	1	1	1	20
Malaysia	10	10	10	20

The main raw materials used

In the beginning was rapeseed or canola. With the high content of the monounsaturated oleic acid (C 18:1) of about 60 %, the rather low level of saturated fatty acids (palmitic and stearic acid <6 %) and also acceptable levels of linolenic acid (C 18:3) rapeseedoil of the 00-variety types appeared to be by chance a rather ideal raw material for the European climate and of reasonable stability expressed by an Iodine Value of <115 [15].

Other raw materials used were palmoil (>40 % palmitic acid - C 16:0) in Malaysia: because of the high temperature climate there no problems were observed with „winter" operability [3]. In Austria, France and Italy traditional sunfloweroil was seen as another option, while soybeanoil became the raw

material of choice in the USA: both oil types have an Iodine Value of >115 due to a content of linoleic acid (C 18:2) of >50 %.

Searching for improved economics and low cost sources of tryglyceride raw materials used frying oils collected at restaurants and households were tested for Biodiesel production with improved process technologies. At the end of the pipe it was high quality standardised Biodiesel and the tests in diesel engines were completed with the same results as for Biodiesel from virgin plant oils [4].

As soon experienced in real life conditions, there was generally the challenge to find the optimum between satisfactory stability (oxidation, polymerisation) and acceptable winter operability as a function of the degree of saturation, mono- or polyunsaturation of fatty acid compositions in the various oilseed plants and as a function of chain length as well.

Key fuel properties of Biodiesel

In testing plant oils as a fuel it was the first lesson to learn, that pure oils, even of fully refined quality, do not fit the modern fast running diesel engine of high efficiency and with a low emission profile. The methyl-esters were the plant oil derivative of choice, rather simple in production and coming very close to the fuel properties of diesel. There are slight but acceptable differences in density and viscosity, the higher flashpoint is a beneficial safety feature, and the sulphur-free plant oil is the reason for the excellent SO_x-emissions of Biodiesel. Although standards do not differentiate the Cetane nr., one can observe a higher value for Biodiesel in general resulting in a smoother running of the engine.

Table 2. Comparison of fuel properties

Physical-chemical properties:	unit	Diesel EN 590	Biodiesel (PME) DIN V 51.606
standard properties:			
density at 15°	kg/m^3	820-860	875-900
viscosity at 40°	mm^2/s	2,00-4,50	3,5-5,0
flashpoint	°C	>55	>110
sulphur	%w	<0,20	<0,01
Cetane nr.		>49	>49
additional properties:			**Biodiesel (RME)**
oxygen	%w	0,0	10,9
caloric value	MJ/dm^3	35,6	32,9
efficiency degree	%	38,2	40,7

Walter, 1992 [5]

Biodiesel (RME) is already a naturally oxygenated fuel with an oxygen content of about 10 % in the molecule, which varies by fatty acid chain length. Oxygen is causing favourable emission levels, but it is also the cause for a 7 % lower caloric value. This would be expected to reduce engine performance, however tests with different engines under a variety of conditions have shown engine performance to

be comparable to that with diesel fuel. A more efficient conversion of the energy of Biodiesel into engine performance during the combustion process makes up for most of the difference.

As already indicated there are requirements for winter operability measured by the Cold Filter Plugging Point (CFPP). Rapeseedoil-Methyl-Ester with a level of saturated fatty acids (palmitic and stearic acid) less than 6 % can be used down to -8 °C without additives; with the elimination of the saturates a CFPP of impressive -36 °C can be reached, which is ideal for alpine regions [6].

Motor oil dilution has been observed also to a larger extent, as some fuel is always transported from the combustion chamber into the motor oil during piston movements. Biodiesel tends to accumulate in the motor oil to a greater extent than diesel because of the more homogeneous nature and higher boiling line of Biodiesel. However higher inclusion levels of up to 20 % did not result in any adverse lubrication effects. There is however the suspicion, that higher levels of methyl-esters of polyunsaturated fatty acids such as linolenic acid (C 18:3) may lead to polymerisation and the risk of formation of sludge in the motor oil.

As indicated by above lines Biodiesel has excellent lubricity properties and is therefore also considered in France to be used as lubricity additive for low-sulphur fossil diesel fuel, which causes otherwise a breakdown of injection pumps. Lubricity properties of methyl-esters of different chain length have not yet been investigated.

Biodiesel standards - a tool for quality assurance

As a condition for a successful market introduction and supportive acceptance by engine producers and endusers as customers a fuel for diesel engines must be specified with carefully selected criteria as a common tool for quality assurance.

The very first standard was the Austrian RME specification ON C 1190 for Biodiesel based on 00-rapeseed-oil of <5 % content of erucic acid and <15 % content of linolenic acid consisting of a combination of criteria relevant to traditional fossil diesel on the one hand and to derivatives of plant oils on the other hand. It was followed by the Austrian Fatty-Acid-Methyl-Ester (FAME) specification ON C 1191 allowing a wider range of triglycerides - virgin or waste oils and fats of plant or animal origin - provided the required quality for Biodiesel is reached and assured.

Other countries followed with similar standards, e.g.: France - French regulation of December 1993, Italy - decree of December 1993, Czechia - final CSN 65.6507 of November 1994; additional efforts can be observed in Sweden - SS 1554 VAE proposal, and the USA - ASTM committee established. [7].

The most complex Biodiesel standard today is probably the German one for Plantoil-Methyl-Ester (PME) as the preliminary specification DIN V 51.606, which is just now in development to the final version - DIN 51.606. This standard has obtained a strong European dimension, as it is the basis for warranties given by major car producers such as Volkswagen, Audi, Mercedes, a.o. all over Europe; the next step already initiated is the completion of a CEN-specification in

near future, which is supported through a mandate given by the European Commission.

Environmental impacts

Tremendous efforts have been put into life cycle analysis exercises in many countries; Biodiesel appears to be one of the best researched products. Trying to summarise one can state, that there is a clear contribution to the desired reduction of greenhouse gases by at least 3,2 kg CO_2-equivalent per 1 kg Biodiesel [8]; those results have been improved since then by lower inputs in raw material production and by more efficient process technology.

It is as well established, that there is a substantial reduction of SO_x-emissions and considerable ones of CO, HC, soot and particulate matter (PM), while there is a slight increase of NO_x-emission, which can be influenced positively by delay of injection timing, as will be shown by my colleagues. Biodiesel appears to be also an ideal complementary partner for the oxicat. It may be interesting for the plant breeder to know, that fatty acid chain length and related oxygen content as well as boiling line have an influence on emission profiles of the various Biodiesel qualities.

Not surprisingly Biodiesel as a plant oil derivative has a very low toxicity as a product: acute dermal and oral LD_{50} in rats is >2.000 mg/kg bodyweight. This is in line with the excellent values for biodegradability of more than 90 % within 3 weeks, advantageous in case of accidents. Additional ecological studies in comparing fossil diesel with Biodiesel when exposed to trout, daphnia, water cress and algae have shown substantial reduction of toxicity risks to those lead water organisms [9].

Marketing strategies

Bringing a new product to the market requires careful consideration of the market conditions and customer needs. Different strategies have been applied so far:

Italy has one of the highest levels of mineral oil tax in Europe, both on diesel fuel and heating oil. Given full detaxation it was therefore a logic step to penetrate the easier accessable market for heating oil.

France has chosen another strategy by delivering Biodiesel to refineries, where it is blended with 5 % to fossil diesel and distributed through the existing system, mainly by Elf, Shell and Total. However the customer is not in a position to identify the difference in the fuel. This strategy is avoiding to build a separate and costly infrastructure and big volumes can enter the market immediately on the one hand, the advantages of Biodiesel are applied only in a diluted way and without visibility on the other hand.

Another blend strategy is tried in the USA, where 20 % soyaoil-methyl-ester are mixed with fossil diesel, mainly because of price reasons. The 80/20 fuel blend

in combination with a catalytic converter has recently obtained EPA certification for the Urban Bus Retrofit programme.

Taking all the benefits of Biodiesel a 100 % and undiluted to the market is the strategy of choice in Germany and Austria. Target applications identified can be environmentally sensitive segments, e.g. water protection on lakes and quality groundwater areas, irrigation pumps, skiing areas, forestry operations, as well as taxis, city buses in smog endangered locations and the „green" driver.

Micro- and macroeconomic considerations

Without going into much detail the key sensitivities in the microeconomics calculation should be highlighted: by far the most important factor in the production is cost of raw material. Oilseeds from set-aside fields have been traded in Europe at acceptable prices at around DEM 300,--/ton so far, cheap used frying oil and other waste oils and fats can improve the calculation. The second most important factor - and often not recognised as such - is the yield in the process, i.e. to what degree transesterifiable triglycerides and free fatty acids are turned into high value methyl-esters; it should not be lower than 99,5 % [10]. And last but not least a reasonable selling price can be reached only with detaxation as exercised in Austria, Czechia, France, Germany and Italy; it is furthermore approved in Spain and Ireland. Investment cost as expressed in depreciation and interest are comparatively a very minor cost but often overestimated.

It is a more complex and difficult exercise to accomplish the macroeconomics calculation. Following a recently published study of the IFO-Munich, which completed an input-output study assuming 300.000 ha of rapeseed in Germany, a Biodiesel production from this acreage would create 5.000 jobs and would justify already 70 % of the detaxation given [11]. Beside this job-creation-bonus one has to calculate a less-global-warming-bonus, which can be calculated with US$ 485,-- per ton CO_2 [12]. For the application in environmentally sensitive areas the relevant less-risk-bonus depending on local risk levels has to be evaluated individually. In addition there is an energy-supply-security-bonus: a rather easy investigation, as a US-governor study calculated the cost of strategic presence in the Gulf with US$ 9,68 per barrel imported fossil oil into the USA [13]. It is becoming again difficult to give a price tag to the renewability-bonus or the cost of replacement of fossil and finite mineral oil supplies.

The future potential of Biodiesel

Some of the development potentials have been already indicated; to mention a few:

- Further improvement of diesel engines, e.g. delayed injection for reduction of NO_x-emission; or high pressure injection for reduction of motor oil dilution thus lowering the risk of polymerisation.

- Expanding the range of cheap raw materials by developing complex recipes of suitable raw material blends according to local conditions thus improving the commercial result of a Biodiesel plant.
- Tailor-made oils for a specific Biodiesel wanted. High-Oleic (HO) rapeseed and sunflower varieties exist already, High-Lauric rapeseedoil is available as well, Low-Linolenic linseed (Linola) may represent another opportunity and does widen the possibilities of oilseeds in the crop rotation. The following table should give an indication to plant breeders for the various possible priorities and options:

Table 3. Options for different fatty acid profiles

Property:	SHORTCHAIN	←	→	LONGCHAIN
Oxygen content	higher	←	→	lower
emissions	*reduced*	☺··PM + soot		*increased*
caloric value	*lower*	performance ☺		*higher*
combustion	*improved*	☺		*worse*
Boiling line	lower	←	→	higher
emissions	*reduced*	☺··HC		*increased*
CFPP	lower	←	→	higher
winter operability	*better*	☺···°C		*worse*
	SATURATED	←	→	UNSATURATED
CFPP	higher	←	→	lower
winter operability	*worse*	°C	☺	*better*
Cetane	higher	←	→	lower
engine performance	*higher*	☺		*lower*
Iodine value	lower	←	→	higher
oxidation stability	*better*	☺		*worse*
polymerisation	*lower*	☺		*higher*

- Increasing the oil yield/ha in order to assure sufficient supply: At least for rapeseed it appears as if we are just at the beginning of a new era in plant breeding and improvements as the oil content will go up from 40 % to 50 % in the seed and oil yield/ha can go up from presently 1,4 ton to 2,9 ton/ha or more. Keeping in mind the tremendous potential of the Eastern European countries willing to enter the European Union we are fortunately in a position to offer an opportunity in the non-food sector, which is oilseeds for Biodiesel, as shown in an example for Germany:

Table 4. Biodiesel potential for Germany

GERMANY		scenario A	1997	scenario B	2020
Total agricultural acreage in ha			12.000.000		12.000.000
Total consumption of diesel in mt			26.000.000		35.000.000
market share for Biodiesel in mt	*)	6 %	1.560.000 ****)	12 %	4.025.000
raw material from					
used frying oil in mt	**)	1,5 %	390.000		390.000
virgin plant oil in mt			1.170.000		3.635.000
oil yield in mt per ha					
today			*1,40*		
in future	***)				*2,90*
equivalent in ha			835.714		1.253.448
in % of total acreage			7 %		10 %

*) assumption
**) as per realistic forecast of Austrian Fat Recycling GmbH
***) forecast of Max-Planck-Institute Cologne
****) as per „European Energy to 2020", report European Commission 1996 [14]

Envisaging the situation in the year 2020 for Germany we can see, that the forecast of the European Commission for needed renewable biofuels and the supply of raw materials for those volumes of liquid biofuels required match each other and can become reality.

References

1. *Proceedings: Improving Market Penetration for New Energy Technologies: Prospect for Pre-Competitive Support.* European Commission - DG XVII, Brussels, Belgium (October 1996).
2. *Draft Directive to the Quality of Petrol and Diesel Fuel.* European Commission - DG XI, Brussels, Belgium (September 1996).
3. A. Schäfer, Pflanzenölfettsäure-Methyl-Ester als Dieselkraftstoffe / Mercedes-Benz. In: *Proceedings Symposium Kraftstoffe aus Pflanzenöl für Dieselmotoren.* Technische Akademie Esslingen, Germany (1991).
4. T. Sams, Use of Biofuels under Real World Engine Operation. In: *Proceedings of the 2nd European Motor Biofuels Forum*, Joanneum Research, Graz, Austria (1996).
5. T. Walter, Untersuchungen des Emissionsverhaltens von Nutzfahrzeugmotoren am Prüfstand bei Betrieb mit RME / Swiss Research Institute EMPA. *Proceedings of the Symposium „RME" - Kraftstoff und Rohstoff.* FICHTE / Technical University Vienna, Austria (1992).
6. M. Wörgetter, Improved Winter Operability with an CFPP-Improved Biodiesel. *Proceedings: 1st International Conference on Standardisation and Analysis of Biodiesel.* FICHTE / Technical University, Vienna, Austria (1995).

7. H. Schindlbauer, (ed.) *Proceedings: 1st International Conference on Standardisation and Analysis of Biodiesel.* FICHTE / Technical University, Vienna, Austria (1995).
8. K. Scharmer, Umweltaspekte bei Herstellung und Verwendung von RME. In: *RME Hearing.* Ministry for Agriculture, Vienna, Austria (1993).
9. W. Rodinger, *Neue Daten zur Umweltverträglichkeit von RME im Vergleich zu Dieselkraftstoff.* Bundesanstalt für Wassergüte, Vienna, Austria (1994).
10. M. Koncar, Criteria for the Development and Selection of Low Cost and High Quality Technologies for Biodiesel. In: *Proceedings of the 2nd European Motor Biofuels Forum*, Joanneum Research, Graz, Austria (1996).
11. M. Schöpe, Economic Aspects of Biodiesel Production in Germany. In: *Proceedings of the 2nd European Motor Biofuels Forum*, Joanneum Research, Graz, Austria (1996).
12. O. Hohmeyer, *Das Solarzeitalter*, Heft 2 (1993).
13. E.C. Ravenal, *Designing Defence for a New World Order.* CATO Institute, Washington D.C., USA (1991).
14. *European Energy to 2020, A Scenario Approach,* European Commission - DG XVII, Brussels, Belgium (Spring 1996).
15. W. Körbitz, Utilisation of Brassica Oils as Biodiesel Fuel. Brassica Oilseeds: Production and Utilisation. CAB International, Wallingford, Cambridge, UK (1995).

Discussion

Introduction by **W Körbitz**: *The Biodiesel Market Today and its Future Potential*

G Oberdörster: You mentioned the different strategies used in different countries with respect to the use of biofuel. If all the available agricultural surfaces in Europe, for example, were used, would there be adequate supplies of Biodiesel, even in the future?

W Körbitz: The first question is: what is adequate? According to the statement of the International Energy Agency, there is a need for all alternative fuels, i.e. natural gas, bioethanol, hydrogen, the fuel cell and Biodiesel. In this respect, Biodiesel is a big product. It will of course not replace all fossil diesel; this is impossible and will remain so in the future. The European study estimates a 12 % contribution for biofuels including bioethanol to total fuel consumption. So if one allows 8 % or so for Biodiesel, this is a large volume. Both the fuel and engine industries would prefer one fuel to two, and two to four, because it makes life easier. I think there are other forces driving us to a bigger, broader scope of alternative fuels.

J Schell: For 12 %, is it known how much of our agricultural surface would have to be used to grow this quantity?

W Körbitz: If 15 % of the arable land were to be set aside—a conservative value— this becomes realistic given the enlargement of the agricultural surface in Eastern Europe.

R Jördens: I represent the German Federal Ministry of Agriculture. The figures Mr Körbitz presented are very optimistic. This is a long-term view. I think it is necessary to start with the situation as it exists in Germany and Europe at the present. My question is: taking the cost of a litre of rapeseed oil on the world market, about 85 Pf, and comparing this with the price of diesel in the world market without tax, about 30 Pf/l, and given the production cost for a litre of rapeseed oil under European conditions, how can the price gap of a factor of 3 between diesel oil and rapeseed oil be overcome?

W Körbitz: Biodiesel cannot be compared directly with fossil diesel using today's microeconomic standards. This is the reason why I mentioned the importance of the internalisation of external cost and benefit. The German IFO-Institute has published a study showing that 70 % of present detaxation advantages

in Germany are covered by standard national economy calculations. I think we are getting closer to that, but it still has to be justified by more accurate work and made visible and transparent to politicians and the public. This, I feel, is very well understood and accepted once it is explained. This is the comparison between Biodiesel and fossil diesel. The comparison presents a challenge as far as agriculture is concerned. There have been wild variations in set-aside land area in recent years. There have been different markets for food oils and non-food oils, although both may come from fields close to each other. Non-foods generally represent an opportunity for agriculture, but agriculture has not yet implemented the tools and regulations to secure the supply of non-food products. An industry cannot be run on stop-and start basis where, for example, the erratic set-aside policy in the European Union starts with 15 % and reduces this to 12, 10, or 5 % after strong use. This is no basis. We have to agree whether or not non-food as a production sector is an opportunity for the future. If it is agreed that non-food is an objective, the regulations assuring continuous production must be introduced.

Session 1:
Engines for Natural and Modified Plant Oil-Based
(*Chairman: Peter Walzer*)

Engines for Natural and Modified Plant Oil-Based Fuels

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Introduction

In the last couple of years car manufacturers like Volkswagen and Daimler Benz have calibrated their serial diesel engines for the use of Biodiesel. In doing so they insist that the porperties of this Biodiesel must comply with the preliminary standard DIN 51606 for Plant Methyl Ester PWW. Table 1 compares important properties of diesel, rapeseed oil and PME.

Table 1. Fuel properties of Diesel, rapeseed oil, RME

	Diesel	rapeseed oil	PME DIN 51606 (June 94)
Cetane No.	51	41	49-59
Density 20 °C (g/cm^3)	0,85	0,92	0,86-0,9
Heating val Hu (MJ/kg)	42,71	37,3	37,2
Viscosity V_{20} (mm^2/s)	4,7	75	6,5-9
Sulfur (%)	0,05	<0,01	<0,01
Oxygen (%)	-	10-12	10-12
Jod nr.	-		115
Glycerides	-		to be defined

As shown in table 1 one can obtain comparable viscosity and cetane numbers upon esterification of rapeseed oil to RME. In addition, there are properties like the oxygen content which help the diesel process to be almost without formation of soot. The particulate emission could be improved, and the very low sulphur content of Biodiesel can make the use of oxidation catalysts (oxycats) more effective.

It is the purpose of this meeting that the speakers should point out directions which they would suggest for further improving the plants. We have agreed that after the paper presentations we will have a common discussion round in which we want to prepare a kind of shopping list of desired properties for plant fuels (see table 1 there). The authors of session 1 will describe operational behaviours and emissions resulting from using rapeseed oil based fuel. They will intend to formulate fuel specifications for even better engine behaviour and potential hazard of the emissions.

The Automotive Industry's Views of the Standards for Plant Oil-Based Fuels

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Abstract

Plant oil methyl ester (PME), produced in Germany from rapeseed oil, has to date been the only alternative fuel produced from plant oil that has been able to acquire a certain standing on the diesel fuel market. The use of this fuel, known as Biodiesel, is associated with a number of environmental advantages. VOLKSWAGEN has consequently decided to promote the use of this alternative fuel by releasing a wide range of diesel-engined vehicles for operation with Biodiesel. Such releases necessitate not only major changes in the materials used throughout the fuel system but also the use of grades of PME which satisfy all the requirements of DIN 51 606.

This publication is intended to highlight those quality criteria to which particular attention must be paid when using Biodiesel and the possible consequences of non-compliance. Water content, glycerine and glyceride content, iodine number, phosphorus content, flash point and low temperature behaviour are of particular significance in this connection. The relatively frequent breakdowns of vehicles in the field which have occurred in the past and been attributed to low quality grades of PME would indicate that improvements in quality assurance during production and distribution are called for.

Market potential of plant oils as fuel

Of the various options for using plant oils as fuel, only the transesterification of rapeseed oil into rapeseed oil methyl ester (RME) has hitherto been implemented in Germany on a commercial scale. In 1996, approx. 80,000 t of this fuel were sold as „Biodiesel" for diesel vehicles. This amounts to approximately 0.4 % of Germany's diesel consumption for road transport.

Since rapeseed is the dominant oil crop in Germany and central Europe, virtually no other plant oils are considered for fuel production. However, in order not to exclude this possibility, the more general term plant oil methyl ester (PME) has been applied to the fuel [4]. Commercially, the fuel is known as „Biodiesel".

In 1996, the area cultivated for „non-food" rapeseed in Germany was approximately 230,000 ha. If the rapeseed grown on this area of land were to be converted solely into Biodiesel, it could potentially replace approximately 1.0 % of diesel consumption of the road traffic. However, a range of other products are based on this raw material, including biodegradable lubricating and hydraulic oils, so in future too, the entire non-food rapeseed crop will not be available for Biodiesel. Moreover, the production of rapeseed for industrial applications is limited, on the one hand, by the very low prices for this non-food rapeseed and, on the other, by the non-quota restrictions in area negotiated within the framework of the GATT Agreement [7].

Scenarios which forecast the maximum degree to which rapeseed oil methyl ester could replace diesel fuel, disregarding economic and agricultural policy factors, come to the conclusion that the upper limit for replacement lies at between 4 and 5 % [6]. One factor which cannot be overlooked when assessing this fuel's market potential is that rapeseed grown on set-aside land is subsidised by the set-aside premium, which currently averages approximately DM 730/ha in Germany. This set-aside premium could fall in future years. Exemption from petroleum tax must be considered a further subsidy. These subsidies amount to approximately DM 1.10 per litre of Biodiesel produced from rapeseed [7].

VOLKSWAGEN product releases for Biodiesel

As VOLKSWAGEN has set itself the objective of providing motor vehicles with a high level of environmental compatibility, these vehicles should make a benchmark contribution to conserving resources and to exploiting the advantages of renewable energy sources, which are in some respects, if not all, particularly environmentally friendly.

With regard to biodegradability and the absence of sulphur, Biodiesel has clear environmental advantages. The production and use of this fuel also results in lower CO_2-equivalent emissions together with lower emissions of particulates, aromatics and polyaromatics in comparison with the emissions from the diesel fuel chain. Lower vehicle emissions can only be achieved by using oxidation catalysts. These advantages must, however, be offset against increased NO_x and aldehyde emissions [6, 8].

There is quite considerable customer interest in this alternative fuel, with vehicles capable of using Biodiesel in demand from both private and public sector customers. A fuel distribution infrastructure is already in place with over 500 filling stations in Germany [7].

For consumers, the environmental advantages are the principal motivation to use this fuel. Use in particularly environmentally sensitive areas such as forest and water conservation areas is of particular significance here.

VOLKSWAGEN has consequently decided to release a wide range of new diesel-engined VOLKSWAGEN vehicles for operation with Biodiesel, see table 1.

Table 1. Biodiesel operation releases for new VOLKSWAGEN vehicles

VOLKSWAGEN	Polo A03	
	Polo Classic A03	
	Golf A3 and A4	Operation Dates of
	Golf Variant A3	Biodiesel Approval
	Vento A3	Differs According to
	Passat B4 and B5	Vehicle Types
	Sharan	
COMMERCIAL VEHICLES	Caddy	
	Transporter/Caravelle T4	
	LT 2	(As at 1.1.1998)

In addition, conversion kits are also on offer for certain older Golf/Vento, Passat and Transporter/Caravelle models with swirl chamber engines, see figure 1.

Only for Swirl Chamber Diesel
Engines with Oxidation Catalyst

Golf A3 and Passat B4, partly B3
1,9 l with 47 kW and 55 kW

Transporter/Caravelle T4
1,9 l with 44 kW and 50 kW
2,4 l with 57 kW

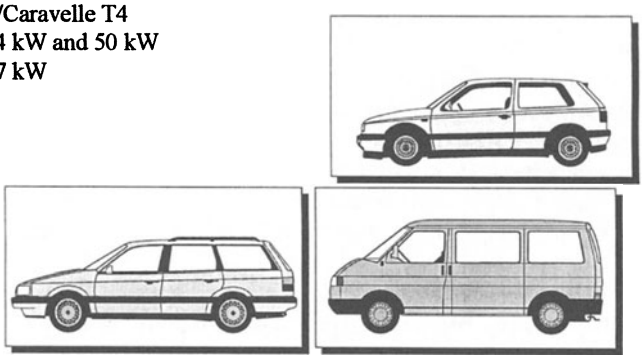

Figure 1. Availability of VOLKSWAGEN conversion kits for Biodiesel operation

Before such releases could be made, materials resistant to Biodiesel had to found to replace numerous fuel system components made from polymers, as plant oil methyl ester attacks the materials previously used [8]. Components which need to be modified in order to ensure resistance to PME are gaiter and seals on the fuel tank sensor, hose material, seals on fuel filter and injection pump, injection

nozzle/injection pump return lines and cap for first injection nozzle. The releases also entail compliance with plant oil methyl ester fuel quality requirements to standard DIN 51 606.

Quality assurance as a precondition for reliable vehicle operation

A precondition for the successful use of plant oil methyl esters as an alternative fuel for car and commercial vehicle diesel engines is that they must run just as reliably as on diesel fuel. If this alternative fuel is to have a long-term future on the market, it must be able to fulfil the same requirements as are currently met by standard commercial diesel fuel, *i.e.* it must be supplied as a high quality product.

Figure 2 outlines the automotive industry's and motorists' most important requirements for plant oil methyl ester. These include compliance with all emission standards, handling characteristics when filling up which match those of diesel fuel and operational reliability of the same high level enjoyed with conventional diesel. In order to ensure that the use of Biodiesel does not generate increased quantities of waste oil, its use in Volkswagen vehicles will not mean shorter oil change intervals. Keeping these intervals unchanged, however, does depend on ensuring compliance with strict fuel quality criteria (to DIN 51 606). Biodiesel must be considered a critical factor with regard to lubricating oil contamination due to its ability to polymerise and its glycerine and glycerides content.

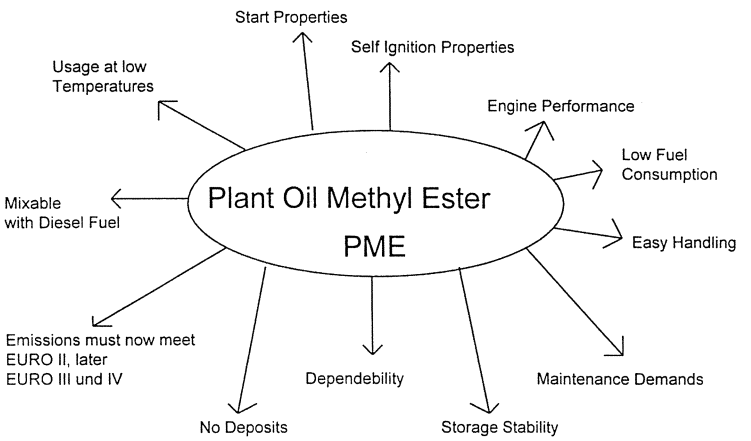

Figure 2. Characteristics in which plant oil methyl ester (PME) must be able to match conventional diesel fuel

Due to the great molecular similarities of plant oil methyl esters to typical paraffinic diesel fuel components, these alternative fuels have every chance of fulfilling the demands that a diesel engine makes of its fuel [2]. This is particularly evident from the following criteria (table 2):

Table 2. Similar molecular structures and properties of PME and diesel fuel

CH_3-O-CO-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2=CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3
(Oleic acid methyl ester)

CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3
(n-Hexa-decane (Cetane))

Comparable to Diesel Fuel	Cetane Number
	Density
	Viscosity
	Heating Value
	Final Distillation Point
Better than Diesel Fuel	Sulphur Content
	Flash Point
	Free of Aromatics
	Biodegradability

Standard DIN 51 606 was developed on the basis of experience gained in numerous research and test programmes with rapeseed oil methyl ester and methyl esters of other plant oils in order to define the quality of such fuels. This preliminary standard encompasses the same characteristics as DIN EN 590 for diesel fuel, but some of the limit values are different [3, 4]. It also specifies some additional characteristics of significance to engine operation with PME (table 3):

Table 3. DIN 51 606 specifications for plant oil methyl ester in comparison with DIN EN 590 specifications for diesel fuel [3, 4]

DIN EN 590 Diesel fuel	DIN 51 606 PME
common characteristics: Density, Viscosity at 40 °C, Flash Point, CFPP, Sulphur Content, Carbon Residue, Cetane Number, Ash Content, Water Content, Particulate Matter, Copper Corrosion, Oxidation Stability, Boiling Range	
	additional characteristics for PME:
	Acid Value
	Methanol Content
	Iodine Number
	Phosphorus Content
	Mono-, Di-, Triglycerides
	Free Glycerine
	Total Glycerine

VOLKSWAGEN's experience to date with Biodiesel grades from various sources has shown that compliance with all the defined limit values is very important in ensuring reliable engine operation and the durability of all components affected by the fuel.

Non-compliance with certain specifications for which limit values are set in DIN 51 606, has frequently resulted in criticism of the grades of Biodiesel offered for sale.

Such criticism mainly relates to the following criteria:

Water content

One significant quality criterion for plant oil methyl esters, which are hygroscopic, is their water content. Unlike diesel fuel, which absorbs virtually no water, Biodiesel can absorb up to approximately 1700 ppm of water at room temperature.

At such high water contents, biological growth may occur in PME. If this phenomenon occurs in a vehicle, it results in engine failure. DIN 51 606 thus limits the water content of plant oil methyl ester to 300 mg/kg. This narrow tolerance range for water is intended to make it unnecessary to add biocides to the fuel to impart resistance to biological growth. The working party which drafted DIN 51 606 decided to limit water content as there was a desire to avoid the use of biocides on environmental protection grounds.

Relatively large quantities of water may result in corrosion phenomena, for example the chromate film on chromated components may dissolve and dezincification of brass components may occur. This phenomenon is associated with gumming, i.e. the Biodiesel polymerises due to the catalytic action of the metallic surface protection, which may lead to blockages in the fuel injection system.

At low temperatures, an elevated water content may result in water separation both in pure Biodiesel and in blends with diesel fuel. Such water separation may result in fuel line and fuel filter blockages.

Table 4 shows the permissible PME water content to German standard DIN 51 606. In the past, grades of Biodiesel were frequently found to have a water content higher than 300 ppm and, in some cases of above 1000 ppm. This led to the demand for production processes which ensure a sufficiently low water content, and for appropriate care during distribution (transport and storage conditions, and duration of storage).

Table 4. Consequences of excessive water content in plant oil methyl ester

☹ Biological growth
☹ Corrosion
☹ Segregation

max. water content in PME	300 ppm	DIN 51606
max. water content in diesel fuel	200 ppm	DIN EN 590

Glycerine and glycerides content

Free glycerine and monoglycerides contain vicinal OH groups which may be highly chemically aggressive towards non-ferrous metals and chromium alloys. If the upper limit for the content of these substances in PME is exceeded, excessive quantities thereof may be transported via the blow-by stream into the crankcase where they come into contact with such materials. This may result in copper, for example, being dissolved out of a non-ferrous metal alloy by complexing reactions. Areas threatened by glycerine and monoglycerides include not only the chromium plating of the compression rings, but especially the materials used in bearings such as the crankshaft bearings, damage to which results in costly engine repairs (table 5).

Table 5. Consequences of excessive glycerine and glycerides contents in PME

☹ Non-ferrous metal corrosion
☹ Corrosion of chromium alloys
☹ Deposits in the combustion chamber

max. free glycerine content	0.02 wt.%
max. monoglyceride content	0.8 wt.%
max. diglyceride content	0.1 wt.%
max. triglyceride content	0.1 wt.%

Di- and triglycerides may lead to deposits on the injectors and in the combustion chamber. They are not themselves critical in non-ferrous metal corrosion, but under certain fuel storage conditions may be hydrolysed by water in the fuel into monoglycerides or glycerine. For this reason, a specific maximum di- and triglyceride content in Biodiesel must not be exceeded. The content of triglycerides (untransesterified plant oils) also indicates how carefully the product was transesterified and purified.

Compliance with the limit values specified in the standard must be ensured during the production process as the content of chemical components with vicinal OH groups can change no further during fuel distribution and storage.

Iodine number

The iodine number is a measure of the number of double bonds in the fatty acids of the corresponding glycerine esters. This parameter also applies to the corresponding methyl esters.

It is known from testing that when diesel engines are operated with plant oil methyl esters having iodine numbers of >115, deposits are formed on injection nozzles, piston rings and piston ring grooves. These effects are attributable to the thermal instability of double bonds, of which there may be several in some fatty acids. If they are heated, polymerisation reactions may occur, resulting in

carbonisation and ultimately in the formation of deposits [5]. If unburnt fuel reaches the lubricating oil, polymerisation may occur on exposure to heat, resulting in the formation of oil sludge (table 6).

Table 6. Consequences of excessive iodine numbers

☹ Deposits in the combustion chamber
☹ Deposits on injector nozzles
☹ Formation of oil sludge

max. iodine number	115 g/100 g	DIN 51 606

On the German market, plant oil methyl ester usually has an iodine number of below 115 as rapeseed oil, which has iodine values of 96 to 110, is virtually the only plant fat used. Some other plant oils have distinctly higher iodine numbers (table 7). The elevated iodine numbers of soya oil and sunflower oil are principally attributable to their high content of diunsaturated linoleic acid (50 to 60 wt.%).

Table 7. Iodine numbers of various plant fats [1]

Fat	Iodine number
Rapeseed oil	96-110
Soya oil	122-128
Sunflower oil	127-134
Olive oil	80-85

Plant oil methyl esters with iodine numbers greater than 115 may only be used as fuel for diesel engines once they have been subjected to hydrogenation in order to reduce the number of thermally unstable double bonds. Such fuels will otherwise result in engine defects.

Phosphorus content

Just like lead, excessive amounts of phosphorus and phosphorus compounds in the exhaust gas result in irreversible damage to the oxidation catalysts in diesel vehicles. The damage is caused by the formation of phosphorus films on the catalytically active surface which prevent the conversion of other pollutant components. The phosphorus content of plant oil methyl esters must consequently never exceed 10 g/kg (table 8) [1, 4, 5]. If such catalyst damage were to occur, it would mean that, in terms of emissions, Biodiesel's potential advantages over diesel fuel could no longer be achieved, and instead increased pollutant emissions could occur, possibly even exceeding limit values. In the interest of reliably

maintaining a high level of pollutant conversion on the oxidation catalysts in the long term, the phosphorus content should remain well below the stated limit.

Table 8. Consequences of excessive phosphorus content

☹ Irreversible defects of catalyst
☹ Contamination of fuel

max. phosphorus content	10 ppm
by way of comparison:	
max. phosphorus content in petrol	2 ppm

Phosphorus content is also an indication of the purity of the grade of PME, as phosphorus usually occurs in the form of mucilaginous substances which ought to be separated from the fuel during the production process. Especially at low temperatures, such mucilaginous substances may result in fuel system blockages.

Flash point

Plant oil methyl esters have a flash point of >100 °C. This is an important quality criterion as values lower than this always point towards contaminations with methanol from the transesterification process or to mixing with diesel fuel or petrol [1].

Unlike diesel fuel, which has a flash point of >55 °C (hazard class AIII), PME may be stored without being subject to particular safety measures, a feature which may be of particular interest to small users with their own fuel storage facilities.

Low temperature flow behaviour

The same temperature limits for low temperature flow behaviour, stated as the CFPP (Cold Filter Plugging Point), are required for PME as for diesel fuel (table 9):

Table 9. Low temperature flow behaviour of PME and diesel fuel

☹ CFPP not sufficient to discribe cold behaviour

CFPP		
15.04. to 30.09.	0 °C	DIN 51 606 and DIN EN 590
01.10. to 15.11.	-10 °C	
16.11. to 28.02.*	-20 °C	
01.03. to 14.04.	-10 °C	

*: in leapyears to 29.02.

Experience has, however, shown that solid PME components may be precipitated even if the specified CFPP values are observed. This may in particular be the case if PME is stored for an extended period at low temperatures, even if these temperatures are still above the lower limit according to the specified CFPP test method [5].

In order to ensure trouble-free engine operation throughout the year, as assessed *inter alia* by low temperature operability, special flow improvers, which like PME should be biodegradable, are required. Moreover, a test method better adapted to PME's low temperature behaviour, which differs from that of diesel fuel, should be used instead of the CFPP method.

Specification of a Biodiesel reference fuel

The petroleum-based diesel reference fuel CEC-RF-03-A-84 must currently be used in order to prove compliance with the legally prescribed emission limit values for diesel engines. This means that standard diesel engines are optimised for this reference fuel. A reference fuel with correspondingly narrowly defined properties is not yet available for Biodiesel.

Although there is no intention of developing modified diesel engines which are specially optimised for Biodiesel, it is nevertheless desirable to define a Biodiesel reference fuel, the properties of which have a much narrower range of variation than that permitted by DIN 51 606. Among other things, this reference fuel would allow the results of emission tests to be more readily compared. Such a reference fuel should correspond to mean values of the properties of commercially available grades from different suppliers [1].

Conclusions

There is only very slight potential in Europe as in Germany for biofuels made from renewable raw materials to replace fossil fuels. Only Biodiesel has so far managed to win a niche market, approximately 0.4 % of the market for diesel fuels for road transport use (table 10).

Table 10. Plant oil methyl esters - summary and outlook

➔Very Small Substitution Potential
➔Positive Ecological Properties Convinced VOLKSWAGEN to Offer Vehicles for PME
➔Quality Demand: at Least DIN 51 606
➔Effective Quality Control Required
➔Definition of a PME Reference Fuel

In the light of the environmental advantages, VOLKSWAGEN has decided to release a wide range of new vehicles for operation with plant oil methyl ester and to offer conversion kits for certain older vehicles.

Such releases are subject to this alternative fuel being able to comply with all the specifications of preliminary standard DIN 51 606. In the interest of motorists wishing to use Biodiesel, all parties involved must ensure that breakdowns will not occur.

In the past, it has repeatedly been observed that Biodiesel grades have been offered for sale which did not comply with the standard in critical respects. This has on occasion caused problems with vehicles in the field. An effective quality assurance system for Biodiesel production and distribution is called for in order to prevent this happening in future.

The issue of a definitive standard governing diesel fuel of fatty acid methylester (FAME) is planned for 1998. In parallel, a FAME reference fuel should also be defined.

References

1. J. Bosse and F. Loock, Qualitätskriterien und Erfahrungen zum Einsatz von Bio-Dieselkraftstoffen (Technische Arbeitstagung Hohenheim 1996)
2. J. Connemann, Biodiesel in Europa 1994, *Fat Science Technology*, vol. **96** (December 1994)
3. Dieselkraftstoff DIN EN 590 (DIN Deutsches Institut für Normung e.V., Beuth Verlag GmbH, Berlin 1993)
4. Dieselkraftstoff aus Fettsäuremethylester (FAME) DIN 51 606 (DIN Deutsches Institut für Normung e.V., Beuth Verlag GmbH, Berlin 1997)
5. Proceedings of the International Conference on Standardization and Analysis of Biodiesel, (Hödl, P., Schindlbauer, H., Fichte Research Institute for Chemistry and Technology of Petroleum products, Vienna University of Technology, Vienna 1996)
6. S.O. Koßmehl, Theses, Beurteilung der technischen, ökologischen und ökonomischen Bedingungen für den Einsatz von Biokraftstoffen im Kraftfahrzeug, Technische Universität Berlin (1995)
7. Jahresbericht 1996 (Union zur Förderung von Oel- und Proteinpflanzen e.V. (UFOP), Bonn 1997)
8. K. Weidmann, Anwendung von Rapsöl in Fahrzeug-Dieselmotoren, ATZ *Automobiltechnische Zeitschrift* **97** Nr. 5 (1995)

Vegetable Oil Fatty Acid Methyl Esters as Alternative Diesel Fuels for Commercial Vehicle Engines

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In addition to describing the current state of knowledge, one of the objectives of the symposium on the subject „Plant oils as Fuels - Present State of Science and Future Developments" was to find an answer to the question as to how plant breeding measures might contribute to providing another market for alternative fuels on the basis of vegetable oils, i.e. help solving problems which currently prevent more comprehensive use of these fuels.

Various ways are conceivable to produce engine fuels from vegetable oils; table 1 shows a schematic presentation of three of these possibilities all of which lead to alternative diesel fuels.

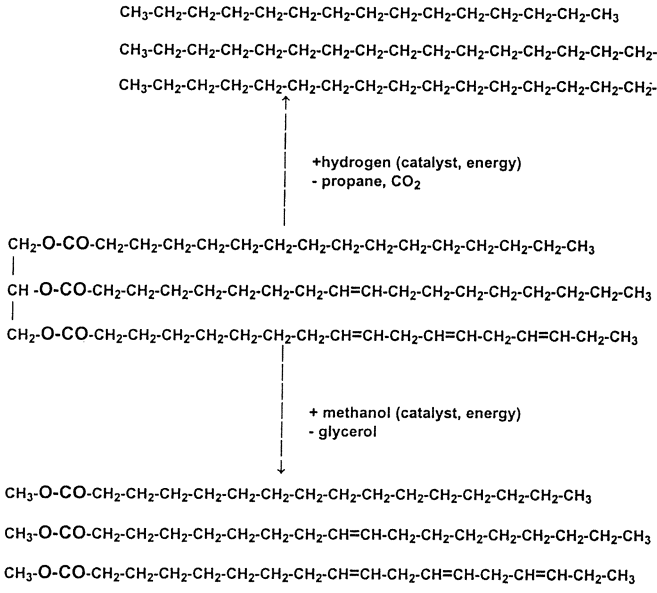
Firstly, there is the chemically unmodified vegetable oil itself which might be used in its pure form or as a blending component to diesel fuel (centre of table 1). (A photograph was presented showing a highly coked injection nozzle after only 30 hours of operation of a conventional engine with a diesel fuel to which only 15 % soybean oil had been added.) The fact that it is impossible to use vegetable oils in existing engines but that dedicated engines would have to be developed for the purpose (if possible at all) makes the chemically unmodified vegetable oils wholly unsuitable from the viewpoint of a vehicle manufacturer.

Another possibility shown in the upper part of table 1 for substituting diesel fuels by vegetable oils which was intensively examined from 1991 to 1994, is to use them as feedstock in processing at the crude oil refinery; despite the many advantages such a fuel would have (no modifications required on engine or vehicle, very good emission characteristics), this possibility is not feasible because of the unsolved problems associated with the cold temperature behaviour.

The only possibility which has been feasible to date is transesterification of the vegetable oils into methyl esters (lower part of table 1).

The similarity of the „Biodiesel" resulting from transesterification with conventional diesel fuel as shown in table 2 explains why it can be used virtually without problems in (only slightly modified) standard production vehicles. A comparison of the fuel-typical properties of Biodiesel and diesel fuel is not suitable to formulate requirements to be met by Biodiesel. Generally speaking, complying as much as possible with the criteria laid down in standards for conventinal fuels cannot be the yardstick to determine the quality of alternative fuels.

Table 1. Possibilities of using vegetable oils as alternative diesel fuels



Refinery processing

Advantages:
- *completely compatible with conventional DF*
- *completely unmodified series production engines*
- very good exhaust gas emissions

Disadvantages / unsolved problems:
- CFPP problem
- processing cost still unclear

Unmodified vegetable oils

(pure or blended with DF)

Advantages:
- local fuel production, short transportation distances
- biologically degradable, "no" processing chemistry

Disadvantages / unsolved problems:
- *not usable in series production engines*
- (small production amounts, sophisticated technology)
- future emission limits achievable with DF and biodiesel?

Vegetable oil esters (RME, "Biodiesel")

(pure or blended with DF)

Advantages:
- biologically degradable
- *series production engines → only small modifications*
- short realisation time (e.g. Germany: 600 filling stations)

Disadvantages / unsolved problems:
- processing cost (appr. 0.10 to 0.15 DM per litre of RME?)

Table 2. Typical fuel properties of rapeseed oil, RME (Biodiesel) and diesel fuel, together with DIN EN 590 requirements

Fuel property	Unit	Minimum acc. to DIN EN 590	Maximum acc. to DIN EN 590	Rape seed oil typically	RME (Biodiesel) typically	Diesel Fuel typically
Approx. chemical formula				$C_{57}H_{101.8}O_2$	$C_{19}H_{35.2}O_2$	$CH_{1.85}$
Molecular weight	g/mole			~ 883	~ 296	120 - 320
Lower heating value referred to fuel mass " " fuel volume	 MJ/kg MJ/l			 36.7 33.7	 37.1 32.6	 42.7 35.4
Density at 15 °C	g/ml	0.820	0.860	0.91-0.92	0.87-0.88	0.83-0.85
Distillation at 250 °C 350 °C 370 °C	 % by vol " "	 85 95	 65 	 0 < 85 < 85	 	
Kinematic viscosity at 40 °C	mm^2/s	2	4.5		4.3	2.5-3.5
Flash point	°C	55		300-330	>100	>55
CFPP 15.April - 30. September 01. October - 15. November 16. November - 28. February 01. March - 14. April	 °C °C °C °C		 0 -10 -20 -10	 >0	 < 0 < -10 < -20 < -10	 < -15 < -15 < -24 < -15
Sulphur content up to 30.09.1996 since 01.10.1996	 % by mass % by mass		 0.2 0.05	 < 0.01	 < 0.01	 0.07 - 0.13 < 0.05
Conradson carbon residue (10 % distillation)	% by mass		0.3		< 0.3	< 0.1
Ignition quality	cetane number	49		> 44	> 55	48-52
Ash	% by mass		0.01		< 0.01	< 0.01
Water content (Karl Fischer)	mg/kg		200		< 300	< 200

With regard to some exhaust gas emissions, the vegetable oil fatty acid methyl esters have significantly more favourable characteristics than conventional diesel fuel (figure 1): unburnt hydrocarbons (HC), particulate matter (PM) and smoke are reduced significantly, whereas carbon monoxide (CO) and oxides of nitrogen (NOx) remain practically unchanged and the volumetric fuel consumption (break specific fuel consumption, BSFC) increases slightly as it has to be expected because of the lower volumetric heating value of Biodiesel.

If an oxidation catalyst is used for emission control, CO and HC practically disappear and the particulate matter (PM) is drastically reduced. The oxidation catalyst is virtually only required because of the odour emission; the question is whether a change in the composition of the vegetable oil might reduce the odour level such that it would be possible to use Biodiesel in a commercial vehicle without an expensive oxidation catalyst.

Figure 2 shows the influence of engine design on emissions (black bar in the middle „Euro 0", right bar „Euro 2" with the same fuel, that is rapeseed oil methyl ester(RME)), and also the influence of the Biodiesel quality (left bar: palm oil methyl ester(PME); black bar in the middle: RME; same engine). It is a remarkable fact that the smoke emission is practically cut in half when changing from rapeseed oil methyl ester to palm oil methyl ester. This extremely favourable black smoke emission with PME leads to major consequences for the engine. As we found out in a large field test of PME in Malaysia, this low black smoke emission has a very positive effect on the lubricating oil: Figure 3 shows that for the entire test mileage of 300,000 to 400,000 km the overall contamination of the lubricating oil is more than twice as high for the diesel-operated buses (black) than for the PME-propelled buses (gray). The same applies to the iron contents in the lubricating oil (figure 4) which is an important criterion for engine wear: the low black smoke emission means less soot in the lubricating oil which in turn - because of the hardness of the soot particles - leads to less abrasion and reduced wear.

Apart from exhaust gas constituents such as soot particles, fuel also enters into the lubricating oil, dilutes the oil and collects in the oil in increasing concentration. The upper half of figure 5 shows that the drop in viscosity of palm oil ester caused by oil dilution does not lead to any serious consequences, whereas under identical conditions RME leads to an increase in viscosity although the concentration of low-viscosity fuel in the lubricating oil rises at the same time. In my view, the only explanation is a reaction of the unsaturated fatty acid esters into higher-molecular products with high viscosity.

We had a closer look at this effect when investigating soybean oil methyl ester: for this purpose, we maintained the ester (black bars in figure 6) and a mixture of 90 % lubricating oil and 10 % ester (gray bars) at a temperature of 110 °C for a period of 30 days and then examined the „disappearance" of monomer fatty acid methyl esters using gas chromatography. A temperature of 110 °C is a typical operating temperature of lubricating oils for engines. If the „disappearance" of stearic acid methyl ester is taken as reference (the respective mathematical equation is written in the caption of figure 6), one can see that palmitic acid methyl ester disappears to a slightly larger extent (slightly higher vapor pressure because of shorter chain length, i.e. slightly higher evaporation).

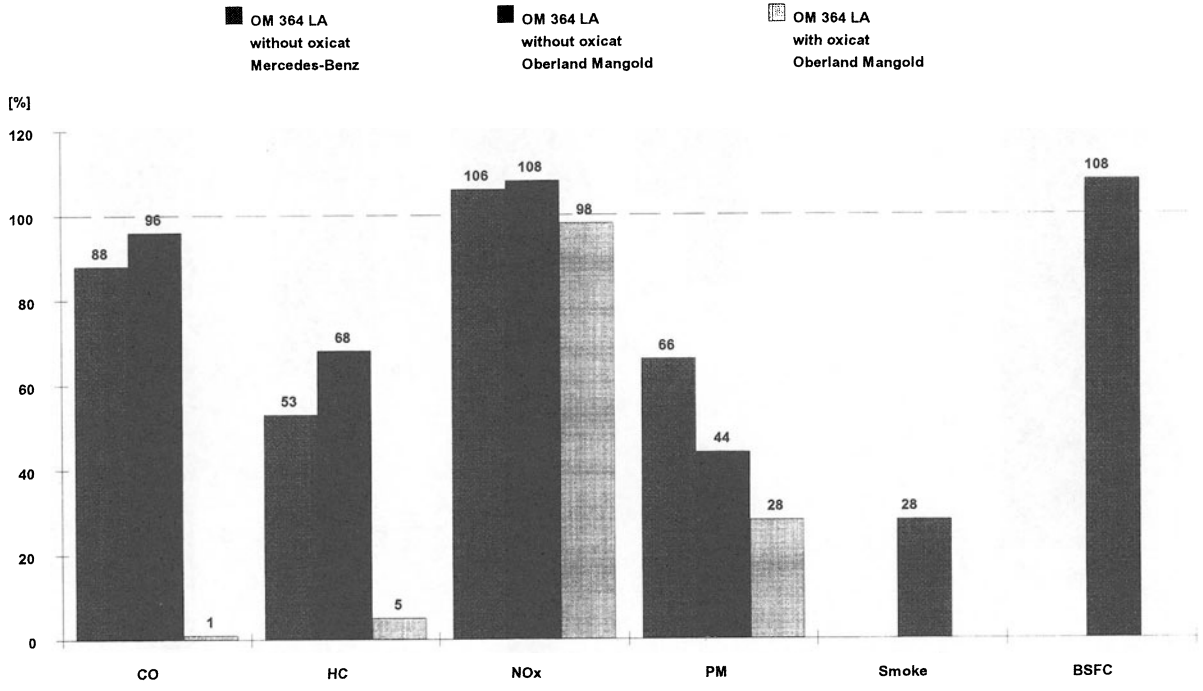


Figure 1. Exhaust gas emissions of a Mercedes-Benz HD diesel engine (OM 364 LA, Euro 2) with Biodiesel operation with and without oxidation catalyst compared to DF operation

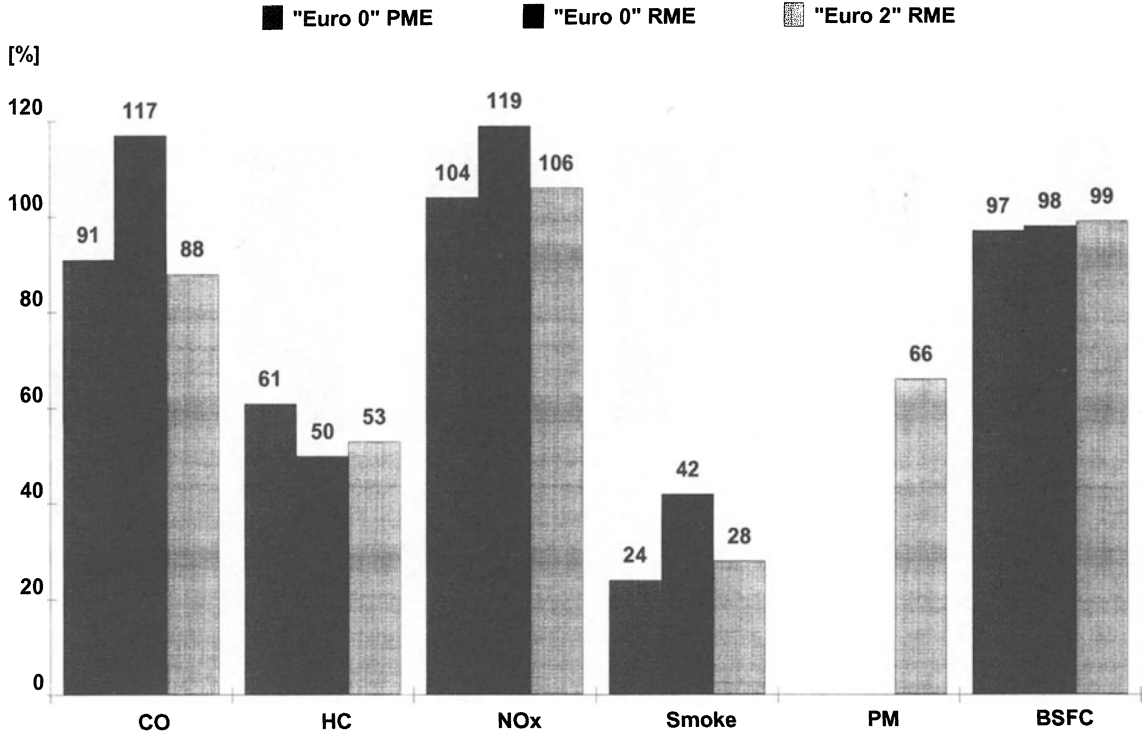


Figure 2. Emissions and energetic fuel consumption of different HD diesel engines using different Biodiesel fuels compared to those with DF operation (100 %) - without oxidation catalyst

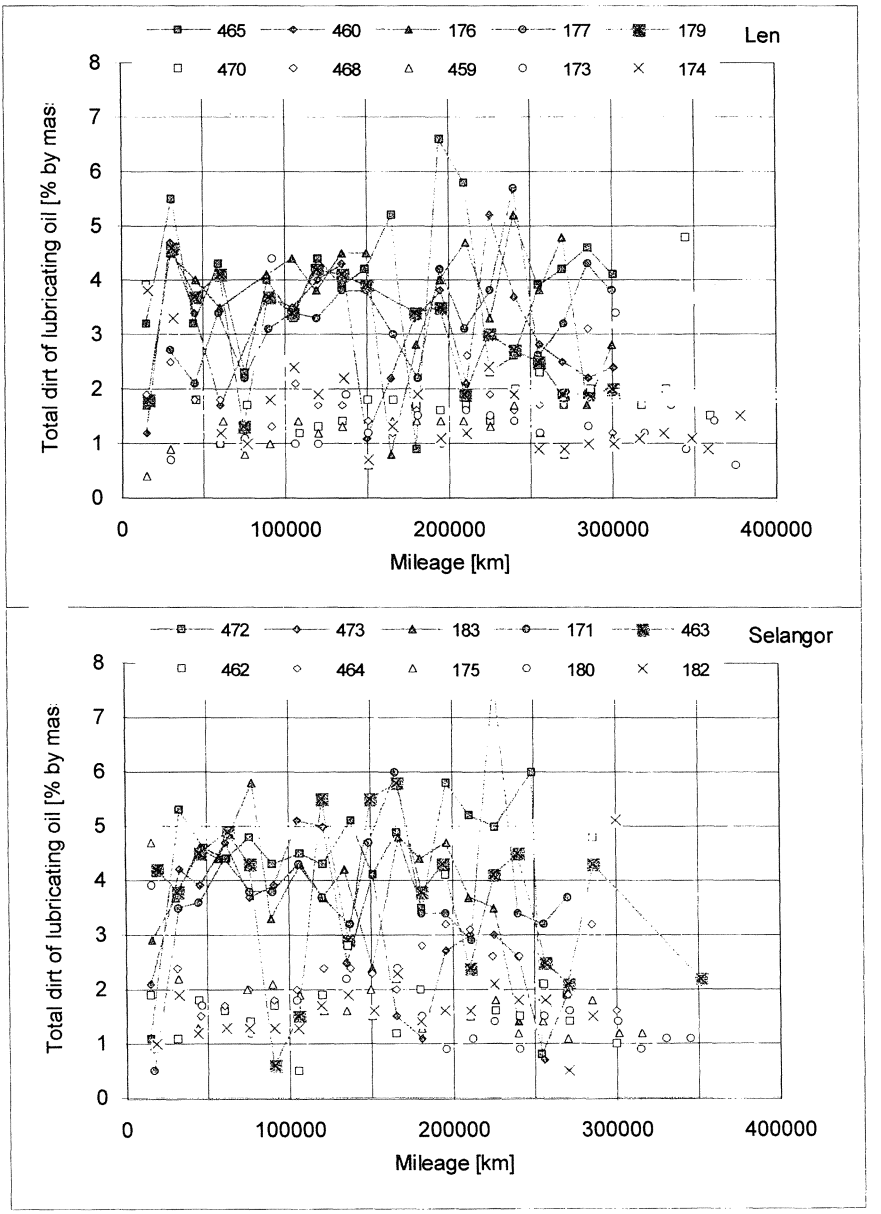


Figure 3. Total dirt of lubricating oil versus mileage for diesel and ester buses

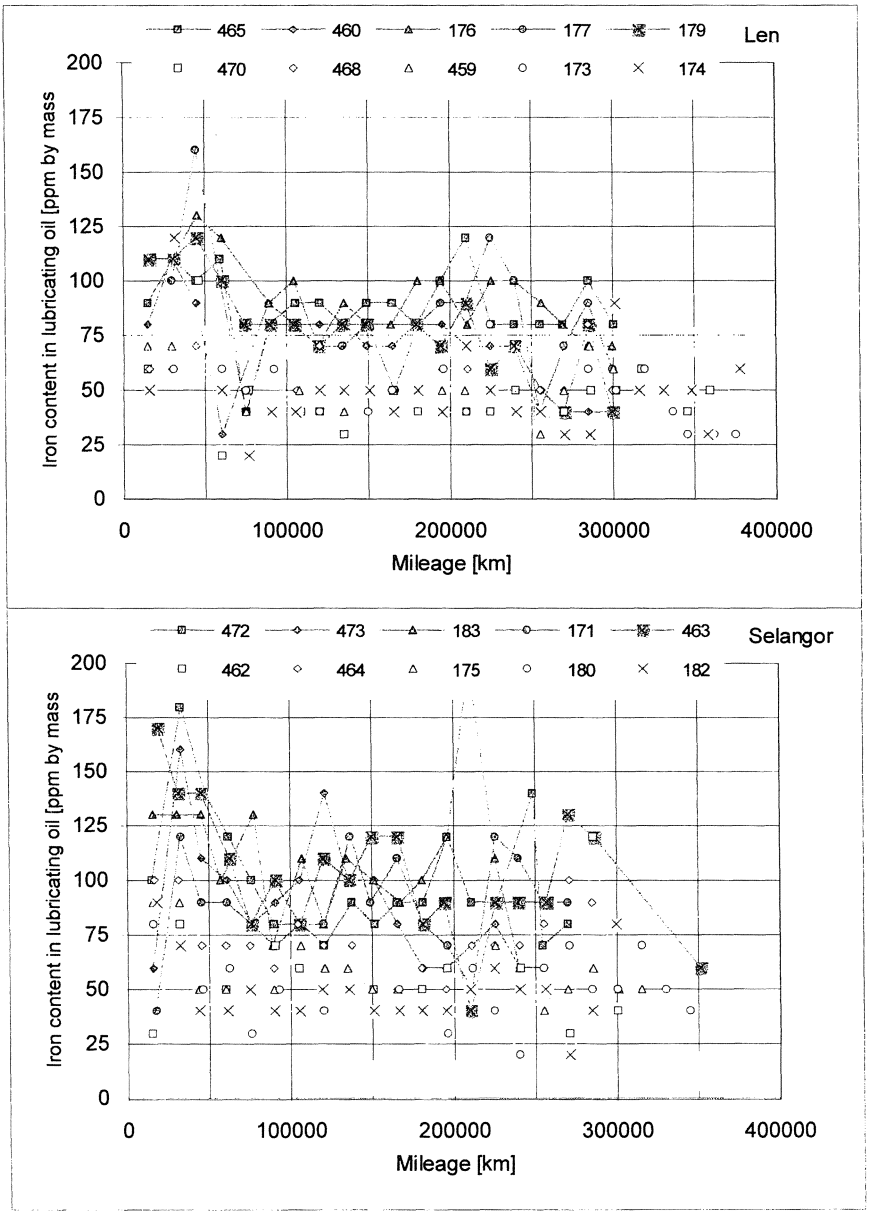


Figure 4. Iron content in lubricating oil versus mileage for diesel and ester buses

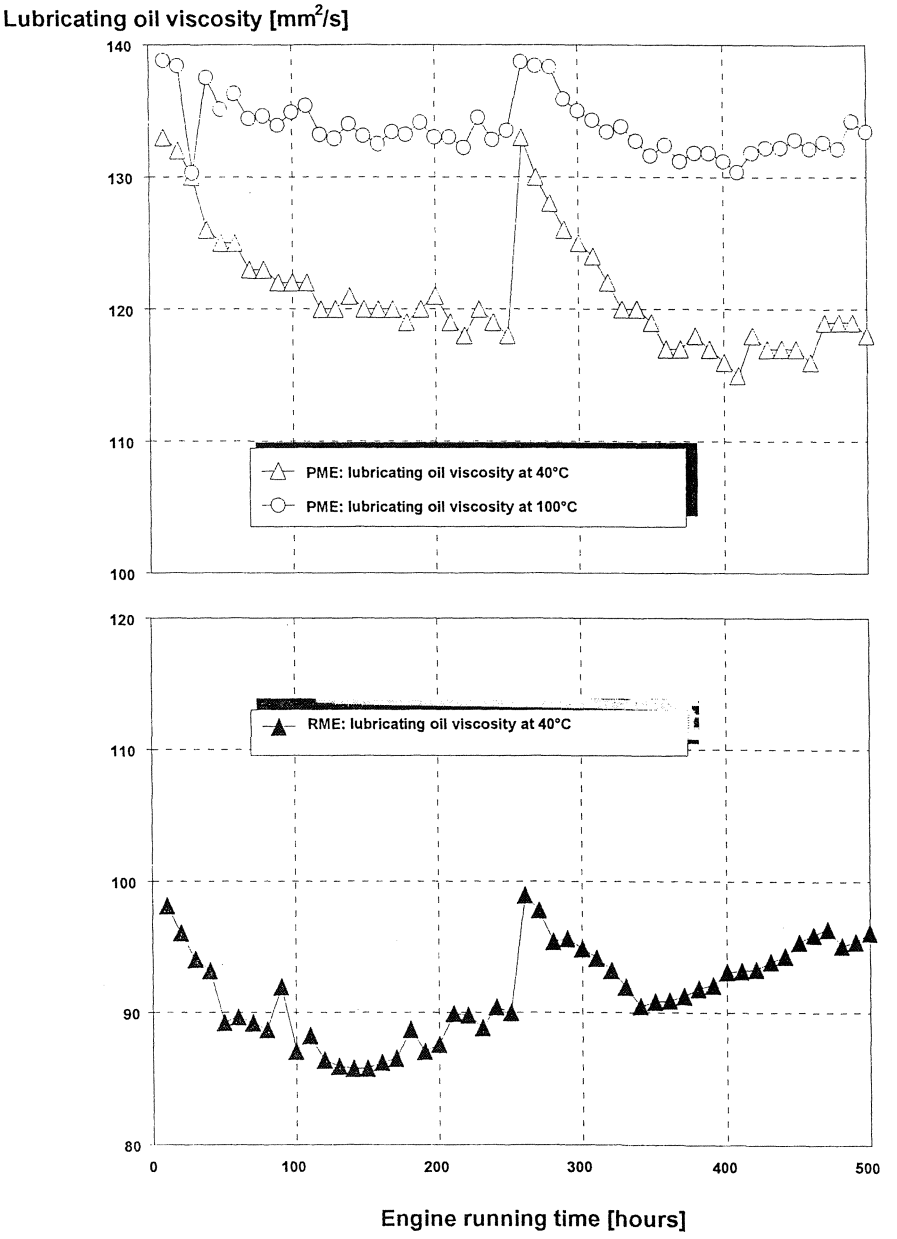


Figure 5. Lubricating oil viscosity with different Biodiesel fuels versus running time

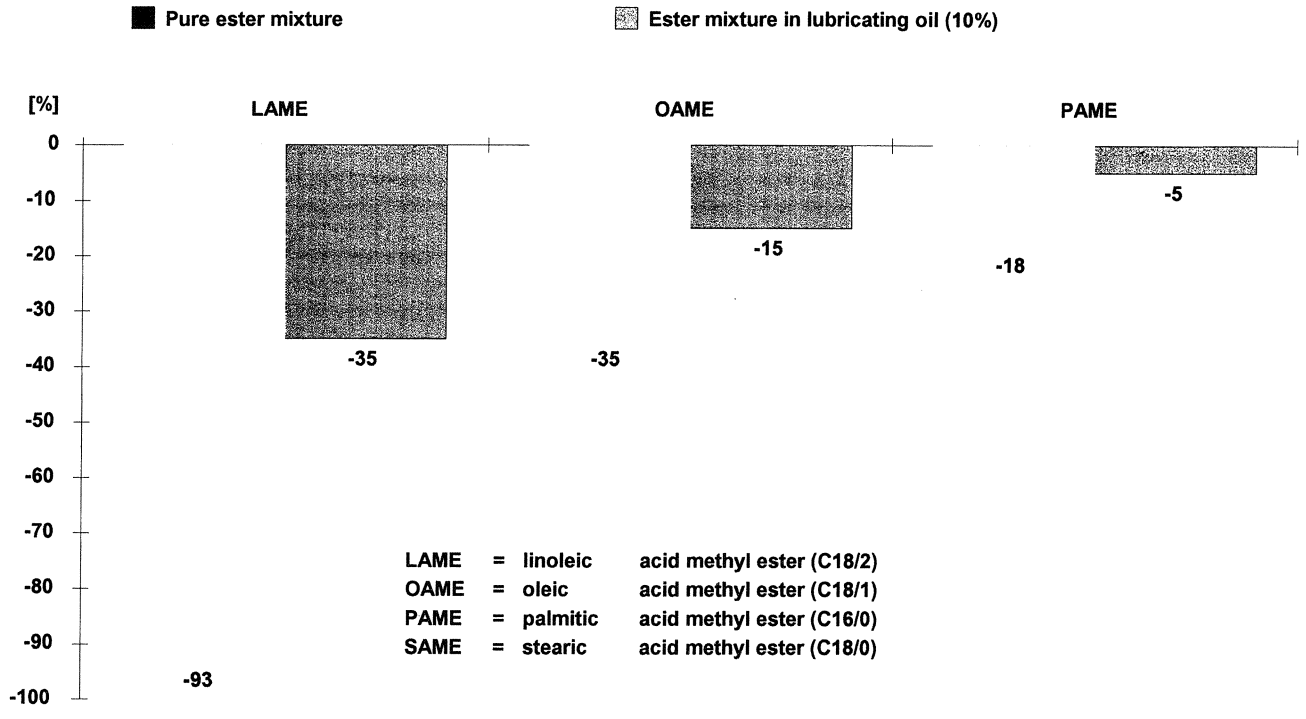


Figure 6. Temperature exposure (110 °C) of vegetable oil esters for 30 days: concentration loss, referred to that of SAME: $[\%] = 100*(1-(c_{XAME,30\ days}/c_{XAME,start})/(c_{SAME,30days}/c_{SAME,start}))$

One can also see that with about the same vapor pressure oleic acid methyl ester disappears to a much larger extent (polymerisation), and that linoleic acid methyl ester disappears to a very large extent, obviously because this doubly unsaturated ester has a much higher tendency for polymerisation than oleic acid methyl ester. With the pure materials, the concentration-dependent effects are (as expected) clearly more distinct than when they are diluted by lubricating oil.

The chemical reactions of the esters in lubricating oil described for the above laboratory experiment explain pretty well the problems found in long-term operation (two photographs were presented showing deposits under the cylinder head cover of a direct-injection commercial-vehicle diesel engine after 250 hours each of continuous operation with various Biodiesels, 250 hours corresponding to the ordinary oil change intervals of these engines.) Operation with soybean oil methyl ester leads to a lot more deposits and a higher degree of sludge formation than RME operation under identical conditions.

Table 3 shows the three vegetable oil esters discussed above (printed in italics) with their main characteristics. A comparison of the endurance test properties for direct-injection standard diesel engines shows a clear-cut graduation: palm oil methyl ester is better than rapeseed oil methyl ester which in turn is better than soybean oil methyl ester. One of the properties which is significant for in-engine behaviour is the degree of unsaturation, i.e. the iodine number. As shown in figure 7, the cetane number which is an extremely important criterion for a diesel fuel correlates with the iodine number as well as with the chain length, i.e. with the molecular weight. This confirms the fact known from the investigation of hydrocarbons which says that the chain length has a favourable influence on the autoignition quality of a diesel fuel, while double bonds have an unfavourable effect.

At this point, I would like to express another request for the composition of Biodiesel: fatty acid esters with carbon chains as long as possible, either saturated or with a maximum of one double bond.

However, this cannot be achieved simply through either removing or hydrogenating the methyl esters with two or three double bonds as we had to learn the hard way in our tests prior to the palm oil methyl ester field test in Malaysia: the Malaysian palm oil methyl ester delivered by ship was solid up to +11 °C and therefore it was not possible to remove it from the containers because it was winter time. In order to have Biodiesel suitable for Western European winter temperatures, shorter-chain saturated fatty acid esters might be „bred into" the Biodiesel. The resulting Biodiesel would correspond to the winter grade Biodiesel as it was prepared by adding 20 % coconut oil methyl ester before flow improvers suitable for Biodiesel were invented. On the other hand, this admixture of short chain fatty acid esters resulted in a significantly more molesting exhaust gas odour so that an oxidation catalyst had to be applied. So, to improve cold temperature behaviour of Biodiesel it seems to be desirable to breed branched chains into the vegetable oil fatty acids.

Table 3. Vegetable oil methyl ester - fuel-relevant properties

Methyl ester of	Number of double bonds	Formula			Composition [% by mass]			Molecular weight [g/mole]
		C	H	O	C	H	O	
Coconut oil	0.08	13.4	26.7	2	73.3	12.2	14.5	220
Palm-kernel oil	0.13	13.9	27.7	2	73.7	12.3	14.1	228
Palm oil	*0.59*	*18.0*	*34.9*	*2*	*76.3*	*12.4*	*11.3*	*284*
Peanut oil	1.10	19.0	35.0	2	77.0	12.2	10.8	296
Cotton seed oil	1.20	18.5	34.6	2	76.9	12.1	11.1	289
Rape seed oil (rich in erucic a.)	1.25	21.0	39.5	2	77.8	12.3	9.9	324
Rape seed oil (poor in erucic a.)	*1.33*	*19.0*	*35.4*	*2*	*77.2*	*12.0*	*10.8*	*296*
Sunflower seed oil	1.49	18.9	34.8	2	77.2	11.9	10.9	294
Soyabean oil	*1.51*	*18.8*	*34.6*	*2*	*77.2*	*11.9*	*10.9*	*293*
Linseed oil	2.12	18.9	33.6	2	77.5	11.6	10.9	292
Diesel fuel, typically	-	**1**	**1.85**	**0**	**86.6**	**13.4**	**0**	**120-320**

Methyl ester of	Iodine number [g Iodine/ 100 g]	Density at 15°C [g/l]	Lower heating value [MJ/kg]	[MJ/l]	Viscosity at 40°C [mm^2/s]	Cetane number
Coconut oil	10	872	35.3	30.8	2.7	63
Palm-kernel oil	15		35.5			
Palm oil	*52*	*872-877*	*37.0*	*32.4*	*4.3-4.5*	*70.0*
Peanut oil	94		37.2			
Cotton seed oil	105		37.0			
Rape seed oil (rich in erucic a.)	98		37.7			
Rape seed oil (poor in erucic a.)	*114*	*882*	*37.2*	*32.8*	*4.2*	*60*
Sunflower seed oil	129	885	37.1	32.8	4.0	61
Soyabean oil	*131*		*37.1*			*56*
Linseed oil	183	891	37.0	33.0	3.7	53
Diesel fuel, typically	-	**830-840**	**42.7**	**35.5**	**2-3.5**	**~51**

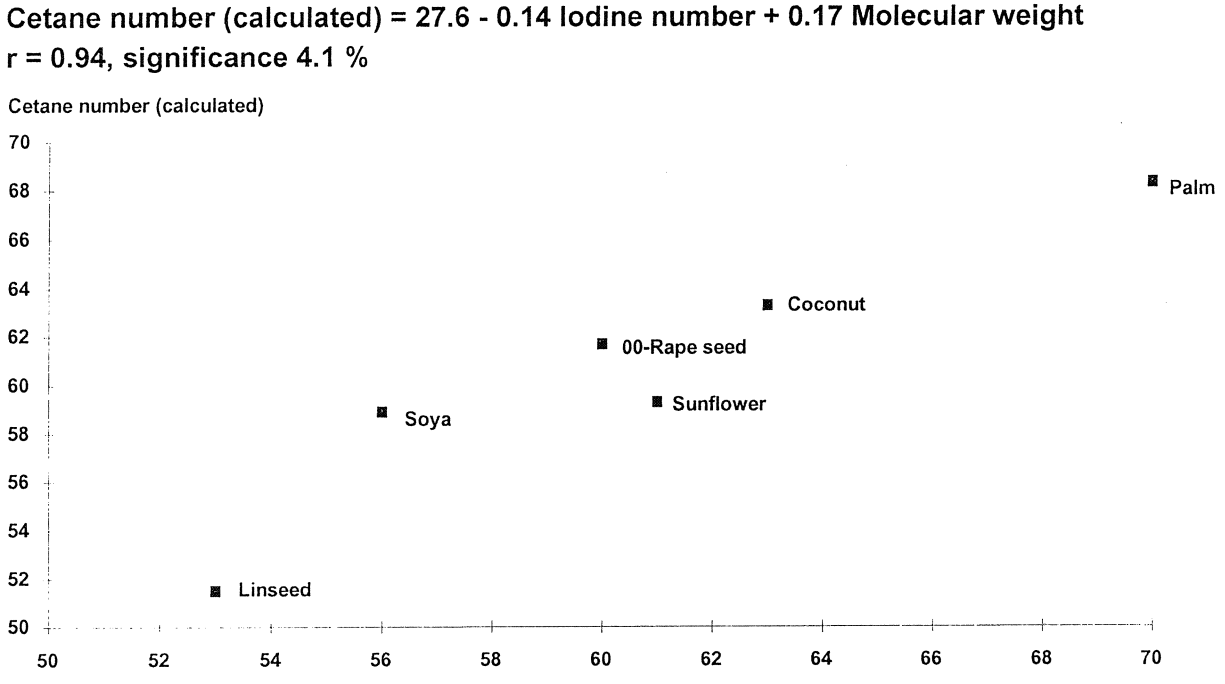


Figure 7. Ignition quality of vegetable oil methyl esters - cetane number depending on iodine number and molecular weight

Table 4. German Biodiesel standard DIN V 51606

Properties	Units	Testing procedure	Limit min.	Limit max.
Density at 15 °C	g/ml	ISO 3675	0.875	0.900
Kinematic viscosity at 40 °C	mm^2/s	ISO 3104	3.5	5.0
Flash point (Pensky-Martens)	°C	ISO 2719	100	
CFPP April 15 - September 30 October 01 - November 15 November 16 - February 28 March 01 - April 14	°C	DIN EN 116		 0 -10 -20 -10
Sulphur	% by mass	ISO 4260		0.01
Carbon residue (10% distillation)	% by mass	ISO 10370		*0.30*
Cetane number		ISO 5165	49	
Ash	% by mass	ISO 6245		0.01
Water	mg/kg	ASTM D 1744		300
Total dirt	mg/kg	DIN 51419		20
Copper corrosion (3 h at 50 °C)		ISO 2160		1
Neutralization number	mg KOH/g	DIN 51558 Part 1		0.5
Methanol	% by mass	to be agreed		0.3
Monoglycerides *Diglycerides* *Triglycerides* *Free Glycerol* *Total Glycerol*	% by mass	to be agreed		*0.8* *0.1* *0.1* *0.02* *0.25*
Iodine Number	g iodine/100 g	DIN 53241 Part 1		*115*
Phosphorous	mg/kg	to be agreed		*10*

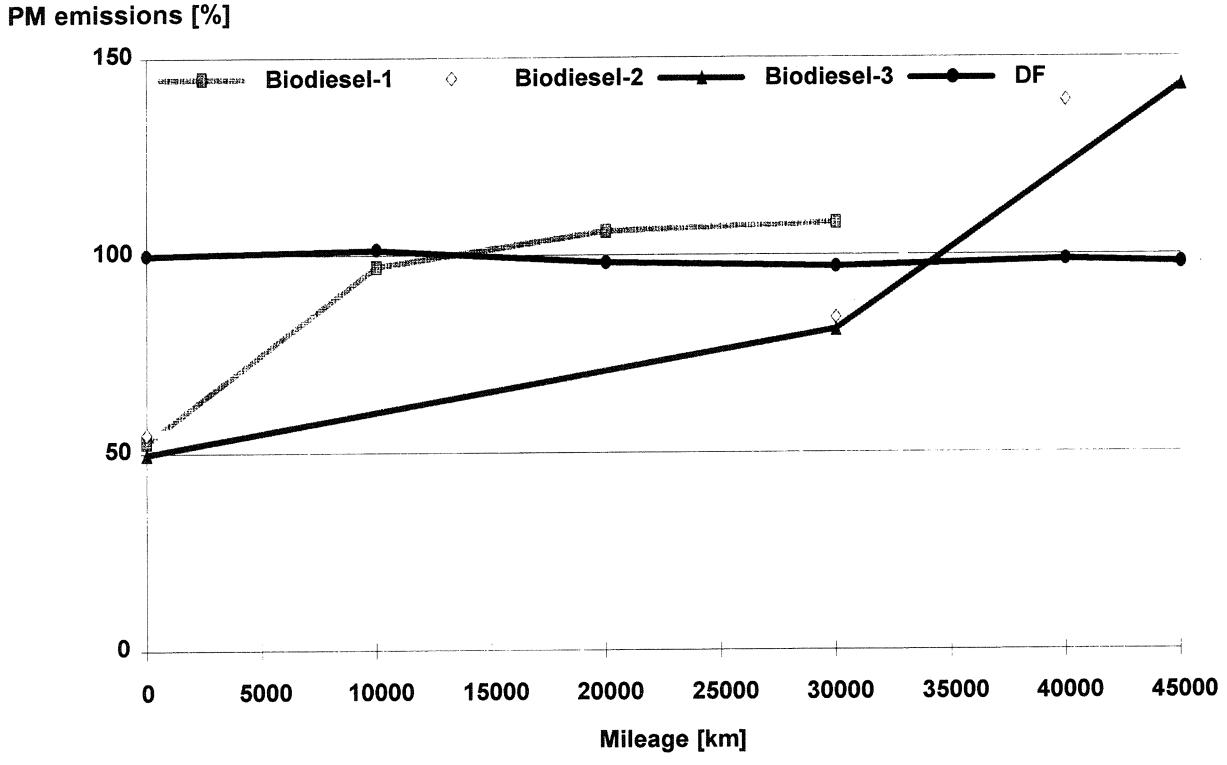


Figure 8. PM emissions of MB passenger cars (OM 601 D20) with RME operation compared to those with DF operation versus mileage: influence of insufficient fuel quality

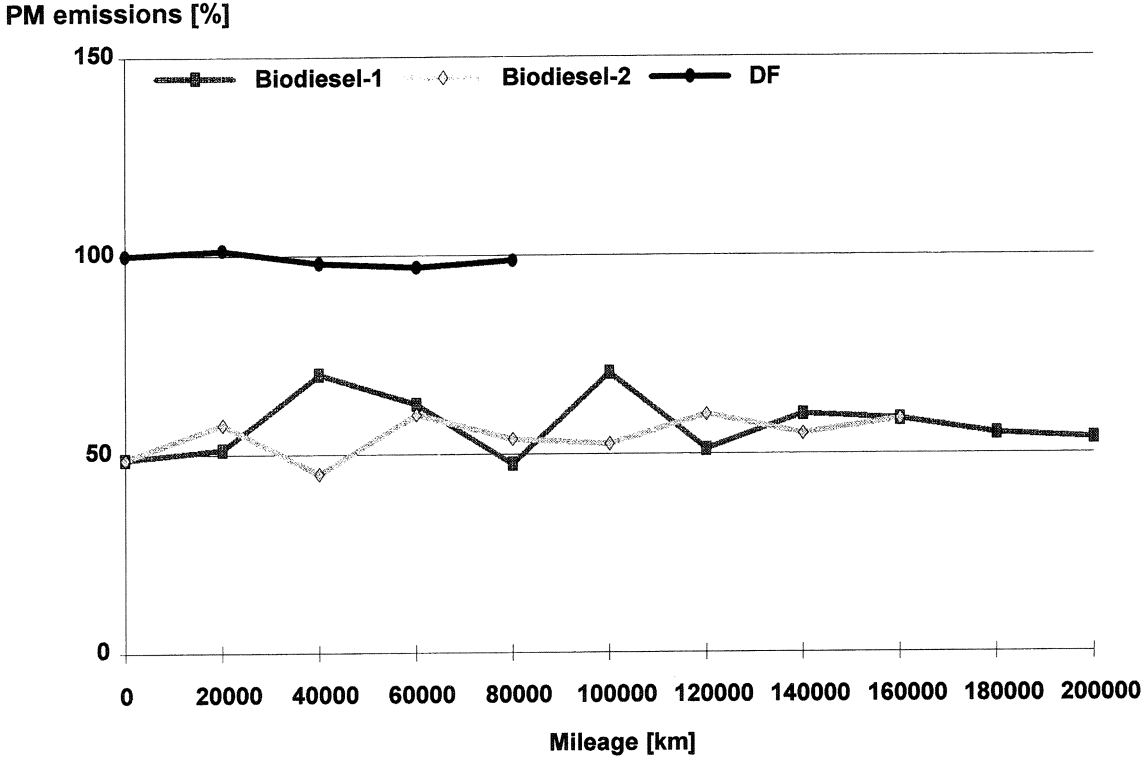


Figure 9. PM emissions of MB passenger cars (OM 601 D20) with RME operation compared to those with DF operation versus mileage: (no) influence of good fuel quality

When we have a look at the currently applicable DIN V 51606 preliminary standard for Biodiesel (table 4), then our quality requirements are already specified, particularly with a view to glycerides and free glycerine, iodine number and phosphorus content (printed in italics). Compliance with this standard is an indispensable must for us and an essential prerequisite for warranty.

The figures 8 and 9 with particulate matter emission results from our Freiburg field test comprising about 30 taxis show the effects of non-compliance of the Biodiesel used with the above mentioned standards first of all on the emissions and subsequently on the service life of the engine. Three of these taxis were taken to Stuttgart on a regular basis and the emissions were measured. Usually, the particulate matter emissions of a diesel engine do not change over the service life of a diesel engine; on the Biodiesel vehicles, however, the particulate matter emissions increased to two or three times the value after short mileages of only 30,000 to 45,000 km (figure 8). It has in the meantime been clearly shown that this is caused by insufficient Biodiesel quality. When the test was repeated in Stuttgart with Biodiesel complying with the standard the phenomenon did not occur again (figure 9).

Table 5. Advantages and disadvantages of using Biodiesel as a diesel replacement fuel

Advantages

☺ Only minor modification needed with existing commercial vehicles, no dedicated engines necessary
☺ Exhaust gas with Biodiesel better than with DF operation concerning smoke, PM and HC and some unregulated emissions (e.g. total aldehydes, carcinogenic PAHs)
☺ Fuel and exhaust gas free of sulphur (a very effective oxidation catalyst can be used)
☺ Based on a renewable energy source, Biodiesel use constitutes a step in the right direction with regard to the greenhouse effect (global warming)
☺ Non-toxic, easy and safe to handle
☺ Fully biodegradable
☺ No water contamination

Disadvantages

☹ Emission of NO_x and some unregulated compounds (e.g. acrolein, benzene) with Biodiesel use not lower than with diesel fuel use
⇨no reason to favour Biodiesel in heavily exhaust emission threatened areas (downtown, underground, etc.)
☹ Lubricating oil dilution in some cases requires reduced oil change intervals
⇨ higher cost, larger amounts of lubricating oil to be disposed
☹ Exhaust gas odour found to be unpleasant
⇨ need for an additional oxidation catalyst
☹ Only small amounts available (worldwide max. 2-3 % of crude oil production)
⇨ no large vehicle amounts to be expected
☹ High cost for the overall economy, otherwise not competitive to crude oil based fuels and natural gas
⇨fiscal incentives necessary

Mercedes-Benz has approved a large number of passenger cars and commercial vehicles for Biodiesel operation; it would go beyond the scope of this paper if I tried to describe the entire volume of approvals. However, one important prerequisite that always applies is a fuel in accordance with DIN V 51606, possibly conversion to Biodiesel-resistant materials and compliance with the operating instructions on the change intervals of lubricating oil, fuel filters etc.

The use of Biodiesel as alternative diesel fuel is associated with manifold advantages, some of which are listed in the upper part of table 5.

Some remaining problems, however, prevent Biodiesel from becoming a full-fledged alternative for every application instead of being a niche fuel. Especially for commercial vehicles, some of the problems are as follows:

- NO_x emissions which make other alternative fuels, for example natural gas, look more favourable.
- Lubricating oil dilution which leads to increased effort and expenditure for lubricating oil and its disposal also on modern engines.
- The exhaust gas odour which is a problem on commercial vehicles. Whereas passenger cars feature an oxidation catalyst as standard, commercial vehicles do not, so that an oxidation catalyst leads to additional cost.
- The low Biodiesel availability world-wide.

We as Biodiesel users cannot answer the question as to whether changes to the vegetable oil raw material, that is plant breeding measures, might contribute to resolving the remaining problems and open questions.

As far as 00-rapeseed is concerned, there seems to be no major need for action for the fatty acid distribution (even though we would like to see the multiple unsaturated fatty acids „bred out") or for the phosphorus content since with sufficient processing quality an adequate Biodiesel quality can be produced.

Should there be increasing pressure for including sun flower oil and soybean oil methyl ester in Biodiesel production, a reduction in the linolenic and linoleic acid percentage brought about by plant breeding measures might be very helpful indeed.

Diesel Engine Technologies for Raw and Transesterified Plant Oils as Fuels: Desired Future Qualities of the Fuels

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Foreword

When reading the title of my paper „Diesel Engine Technologies for Raw and Transesterified Plant Oils as Fuels; Desired Future Qualitites of the Fuels" you might have asked yourself: „Why two subjects for one paper?"

Well, the explanation is simple:

The link between these two subjects is so close and they are so mutually dependent that an overview of both fields is necessary to convey a tangible overall impression which is as realistic as possible and can be understood even without specialist knowledge [1].

Introduction

First of all a few fundamental considerations:

Our modern mobile society requires an enormous interchange of goods and information in its specialisation. Nearly 90 % of goods transport traffic is achieved using diesel engines.

Diesel engines belong to the group of heat engines which - as their name states - convert thermal energy into mechanical energy. In practice diesel engines achieve this with highest efficiency because (with limited maximum pressures) their working cycles give the best approach to the theoretically best conversion process. Therefore they are the most economical of all existing heat machines. This is the main reason for their widespread utilisation which is likely to increase still further as fuel prices increase. So let us firmly bear in mind the fact that diesel engines are the most economical of all existing kinds of heat machines.

They are 100 years old. Rudolf Diesel developed his engine in the MAN factory in Augsburg during the years 1883-1897. Right from the start he envisaged as the goal to devise „the most economical heat engine" [4].

Although diesel engines can operate with fuels in all physical states - gaseous, liquid and solid - liquid fuels are usually the most convenient for practical transportation systems. They are easy and safe to store, i.e. their (chemical) energy

is - in contrast to electrical and thermal energy - easy to store and transport, simple to handle and the energy density is high. Therefore liquid fuels are generally the most valuable form of energy. Regrowing renewable liquid fuels consequently is the most valuable kind of alternative energy [2].

Rudolf Diesel initially used fuels based on mineral oil, preferably petroleum. However, the visionary very soon realised the possibilities offered by fuels from plant sources. Already at the Paris World Fair in 1900 a small diesel engine was operated with peanut oil, and his patent document in 1912 states:

> The use of plant oil as fuel may seem insignificant today, but in the course of time such products can become as important as petroleum and coal tar products are today.

I cannot here go into the overall business economics situation of today, so let me just point out that regrowing plant fuels contribute towards the solution for serious present day problems [2].

- Environmental pollution
- Securing availability of resources
- Agricultural over-production
- Unemployment

The combination:

- most economical heat machine and
- best alternative fuel

is virtually ideal as seen against this background.

Engine concepts for raw and transesterified plant oils

Basic principles

As has already been pointed out, piston-driven engines, in particular diesel engines are generally the thermodynamically most efficient heat engines because:

- the thermodynamic process cycle most closely approaches the theoretical ideal process,
- the pressure and temperature level of the energy conversion can be very high by virtue of the discontinuous (cycled) operation, highest temperatures giving highest conversion efficiency,
- in contrast to flow machines (turbines), the energy conversion efficiency does not depend much on the engine rotation speed and mechanical load.

The chief problem with piston machines with „internal mixing", i.e. especially for diesel engines, is the necessary „instantaneous" but nevertheless thorough mixing of vaporised fuel and combustion air. Because of the cycled operation, only a few milliseconds are available for this mixing procedure which has to take place in a very confined space above or even inside the piston [3].

You might now be asking whether this „torturing" in a very small space inside the cylinder is really necessary. Yes, it has to be so. The revolutionary idea of Rudolf Diesel was not to suck-in a ready to explode mixture of fuel and combustion air, as in gas or carburetor engines, but instead to draw-in just air. Air can be compressed to a much greater extent than is possible with an ignitable mixture (which might thereby tend to explode spontaneously). By drawing-in only air the whole process can be raised to a very high pressure and temperature level giving, as already pointed out, an otherwise unobtainable energy conversion efficiency [4]. The fact that no separate ignition system is required at this high temperature to initiate the combustion process is a welcome side effect of the diesel principle. It is not the basic principle, even though it has led to the generic designation of „self-igniting engines".

Right from the start, i.e. since 100 years, this mixture production, especially with the aim of preventing soot generation, has been the central theme of diesel engine research and development. In spite of great progress especially in recent decades, above all with new fuel injection systems, the injected droplets still do not bring the desired homogeneous mixtures [5]. The mixture remains heterogeneous, i.e. non-uniform [6]. We will appreciate later in this lecture the considerable importance of chemically bound oxygen in the fuel, characteristic of all plant oil fuels.

Methods for producing the mixture

On account of the extremely difficult situation described above, many very different methods for producing the mixture have been devised in the course of time. They require correspondingly different designs for the „combustion spaces" between the piston and the cylinder head. Figure 1 shows the chief methods:

Engine concepts

The various different mixture production and combustion systems partly lead to very different engine concepts. Speaking quite generally, this is due to differing requirements of the combustion process imposed on the participating and neighbouring components.

There are two fundamentally different approaches for using plant oil fuels [1]:

- Adaptation of the engines to the plant oil fuel
- Adaptation of the plant oil fuel to the engines

Adaptation of the engines to „raw natural state" plant oils

Here again there are two very different concepts (see figure 1):

- Chamber method (IDI = Indirect Injection)
- Partial wall acceptance method (CDI = Controlled Direct Injection) with single hole injection nozzle(s)

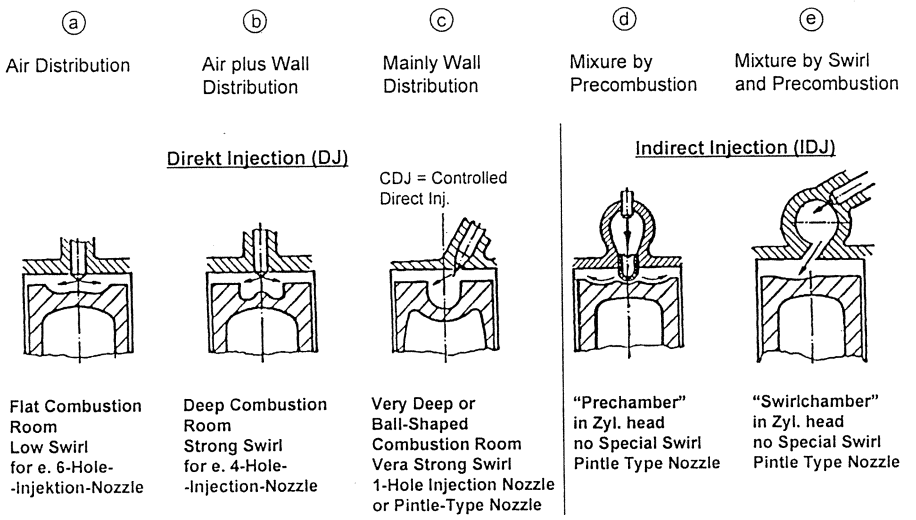
ⓐ
Air Distribution
ⓑ
Air plus Wall Distribution
ⓒ
Mainly Wall Distribution
ⓓ
Mixure by Precombustion
ⓔ
Mixture by Swirl and Precombustion
Direkt Injection (DJ)
CDJ = Controlled Direct Inj.
Indirect Injection (IDJ)
Flat Combustion Room Low Swirl for e. 6-Hole--Injektion-Nozzle
Deep Combustion Room Strong Swirl for e. 4-Hole--Injection-Nozzle
Very Deep or Ball-Shaped Combustion Room Vera Strong Swirl 1-Hole Injection Nozzle or Pintle-Type Nozzle
"Prechamber" in Zyl. head no Special Swirl Pintle Type Nozzle
"Swirlchamber" in Zyl. head no Special Swirl Pintle Type Nozzle

Figure 1: Diesel fuel mixture systems

A main problem when using natural state raw plant oils as engine fuel is that when such fuels are utilised in modern present-day so-called multi-jet direct injection engines, coke deposits are formed on the fuel injection nozzles, pistons and piston rings. This is a consequence of the very high boiling points and the cracking tendency of raw plant oils.

- The old conventional chamber method masters this problem [7] by exploiting the enormous vortex energy in a precombustion or vortex chamber (hence the designation IDI = indirect injection) to enforce complete combustion of the intermediate products or at least to prevent them from being deposited on pistons, cylinder liners and rings. This is successful only for the larger sizes of chamber engines, as for larger automobile and tractor engines [21, 7].

  These methods are rugged, produce relatively little acoustic noise and quite favourable exhaust gas emissions. But they also have a considerable disadvantage: Blow-out from the pre-combustion chamber through relatively narrow channels and with wall losses in the (cooled!) chamber costs a lot of energy. Other disadvantages are the limited supercharging possibility (i.e. limited power in practice) and the relatively large heat dissipation (cooling system!) due to the hot combustion chamber in the cylinder head. Therefore such engines have increased fuel consumption of about 12 to 20 percent. This is becoming less and less acceptable in relation to the energy saving efforts so important today.
- The CDI method (CDI = controlled direct injection) avoids these disadvantages. Here the fuel - as in all modern engines - is injected directly into

the combustion chamber (hence the designation „direct injection"). But the mixture is not produced - as in modern serial production engines - with numerous injection jets operating at very high pressure whereby the fuel atomises and vaporises in the hot combustion air. Instead, one or two relatively compact fuel jets are injected into a restricted spherical combustion space in the piston.

Mixing takes place partly spontaneously in the highly agitated air in the „sperical combustion space", and partly the fuel is briefly deposited on the wall of the spherical combustion space from which it evaporates in a „controlled" manner after a certain delay.

With this method - the same as with the chamber engines - hardly any partially burnt fuel appears so that by-products are not deposited on the cylinder wall. So coking problems do not arise with this method. A contribution to non-coking is also made by the special cast iron shuttle shaft pistons which have high surface temperature promoting fuel evaporation. They nevertheless provide good sealing against the hot combustion gases and their undesired freight by virtue of very small clearance between the piston and the cylinder liner. This provides significant relief for the piston sealing rings which are always subjected to very high stress.

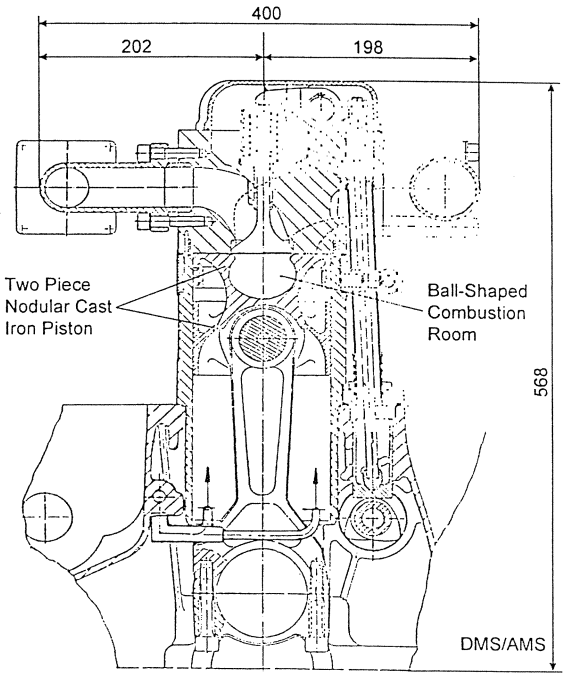

Figure 2: Profile cross-section of a plant oil engine form AMS (Antrieb- und Maschinentechnik Schönebeck) similar: TMW (Thüringer Motorenwerke)

The method was developed for serial production by MAN in the 1950's. Already at that time it was given the name of „salad oil engine" because of its tolerance for a very wide range of different fuels. Later it was further developed by some smaller companies (Elsbett, TMW, AMS) for natural state raw plant oils [8, 9]. Figure 2 shows a cross section of such an engine in which the characteristic spherical combustion space in the piston is easy to recognise.

So these engines can run without problems on natural state raw plant oils (apart from a few restrictions, e.g. with respect to the phosophorous content), whereby the power efficiency is almost as good as that obtained with modern serial production engines today. But their construction and also turbocharging is more complicated, they are somewhat larger and, on account of the high viscosity of plant oils, they require either a fuel preheater or a switchover device for a second fuel circuit with Petro-diesel for small loads and stillstand. Furthermore, the mixture production process depends relatively strongly on the engine rotation speed and load because of the high activity component of the (rotation speed dependent!) air spin. This can lead to some restriction on the possible utilisation of the vehicle under continually changing load and engine rotation speed conditions. However, such engines are very suitable for stationary machine units with constant rotation speed such as block heating power stations, pumps, etc.

DI engines (Multiple jet direct injection engines)

For clarity I must here point out at the outset: After what I have said above, such engines are unsuitable for operation with raw plant oils with cylinder sizes as customary for motor vehicle engines in present day serial production construction. Polymerisation, and above all cracking processes, lead relatively quickly to deposits and coking. However, the better knowledge of reaction kinetics and cause/effect relationships available in the meantime, and some first pilot projects test made, make it seem likely that appropriately designed multi-jet direct injection engines could well be developed for running on raw rape seed oil. A rather large stroke ration ($s/d > 1.5$) would be necessary on account of the necessary high compression values and to protect the cylinder wall against fuel jets.

Such engines would be more difficult to build. They would be larger, heavier and more expensive than present day serial production vehicle engines. At least with respect to fuel injection, mixture production and combustion they would largly constitute a quite new development line. On the basis of the present stage of knowledge it is hardly possible to estimate whether this effort would be worthwhile in comparison with CDI and chamber engines. A conceivable approach could be a long stroke version of a modern serial production vehicle engine using partly the same components.

Adaptation of the plant oil fuel to the engines

Adaptation of the plant oils to the requirements of modern serial production engines (multi-jet direct injection engines) is achieved with a simple chemical process called transesterification. This produces plant oil methyl ester, PME, a

diesel fuel on the basis of plant oils which is eminently suitable for present-day engines of the latest construction. I will come back later to consider PME in detail.

Does this mean that we don't need a different engine concept to run on this kind of fuel? Indeed, we don't need a different concept! It is a great advantage that this fuel can be used with virtually all engines which are being built and used at present, almost without any restrictions. The reservation „almost" is here confined to a few older engine types whose soft components (gaskets, hoselines) are not always inert with respect to PME, but these materials can easily be replaced by PME-resistant alternatives.

Operation and emissions with unchanged engines running on „Biodiesel" are about the same or even considerably improved [10] compared with fossil diesel fuel (Gas Oil, Petrodiesel). Nevertheless, it is pertinent to ask whether this operation of unchanged engines with PME-diesel, in spite of its evident advantages, is already the achievable optimum. Of course, present day serial production engines have been developed for optimised performance with fossil diesel fuel since 100 years. It can hardly be expected that this is also the optimum configuration for a fuel with totally different structure such as Biodiesel. In spite of excellent performance as it stands, this combination of engine and fuel is not the optimum.

NO_x emission can be reduced by 50-70 % [11] with an appropriate, relatively slight adaptation of a modern high pressure fuel injection system, essentially involving only a setback of the start of injection. A still greater reduction is likely possible with additional fine tuning of mixture production and fuel injection course control as well as particular using exhaust gas recycling-systems, oxidising catalysts and eventually Continuous Regeneration Traps (CRT-systems, no sulfur acceptable!). It is at present still not clear to what extent Denox catalysts would be appropriate in addition.

Considering all advantages, including CO_2 savings, it is evident that vehicles with power drive systems optimised for running on Biodiesel produce the smallest environmental pollution.

Desirable future characteristics of plant oil fuels

General estimates and relationships

For 100 years diesel engines have been developed for optimum utilisation of fossil diesel fuels. Apart from some refinements (less sulphur, better ignition capability, etc.), the „gas oil" remained essentially unchanged throughout this time. The increasingly stringent statutory requirements for environmental protection lead to correspondingly stricter emission regulations which cannot be fulfilled, or can be fulfilled only with great effort, by engine modifications alone. This accentuated the need to further develop the second partner (actually the first partner) in the energy conversion process, namely the fuel [3]. Unless

considerable effort is invested, the possibilities are naturally very limited with fossil fuels.

With plant fuels the possibilities are much broader through breeding [20], gene technology, plant selection, production, process steering, blending, post-treatment and with additives. These factors permit development of a wide range of modifications of the plant oils to optimise them for use as engine fuels [2].

An obvious first approach would be to run optimising experiments with all conceivable mixtures of fuels. However, we already know that interactive effects of doing this are extremely complex so that a final assessment of the global results may involve very lengthy investigations. So the initial excitement about simple fuel mixtures has long since vanished. In most cases the aim was to make raw plant oils suitable for use in modern serial production engines (multi-jet direct injectors) without resorting to transesterification allegedly requiring considerable effort.

These so-called three-component fuels (although they usually contained more than three ingredients) consisting, for example, of mixtures of RME, ROR (rape oil, raw), alcohol, petrol, ignition accelerators, etc., have so far not proved to be successful. Apart from engine operation problems, it is hardly possible to maintain an adequately high flashpoint or to comply with acceptable exhaust gas emission limits. For example, figure 3 compares the PAH emissions of such a „three-component fuel" [12]. The situation is similar with some other emissions.

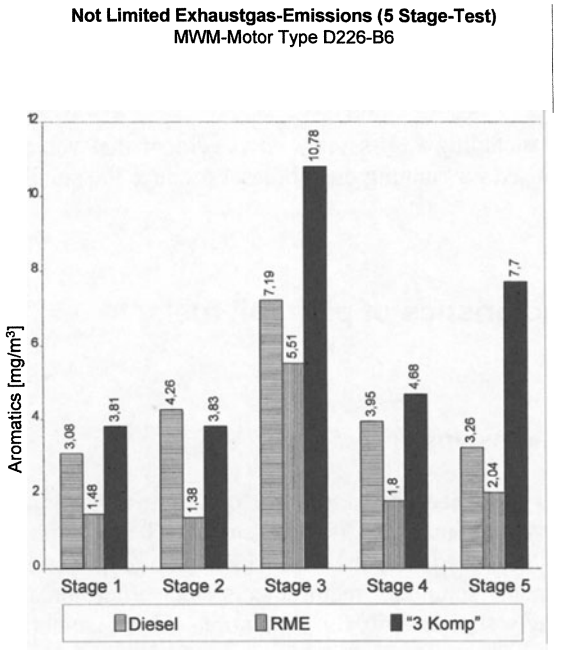

Figure 3: PAH emissions of gas oil, Biodiesel, and a „3-component fuel" in 5-stage test (not limited emissions; measurements TÜV Bayern, source: Maurer/Reiser, Univ. Hohenheim, 1993)

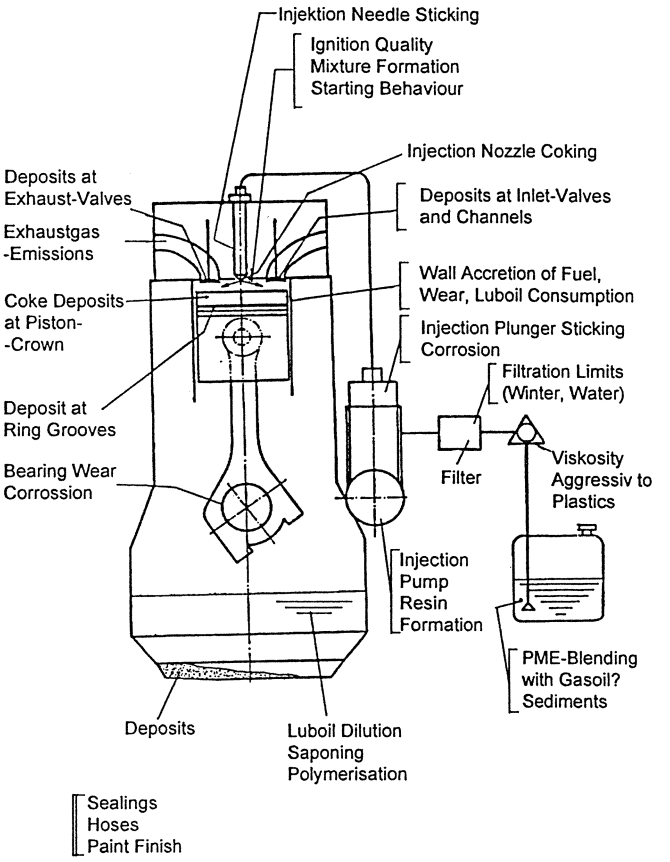

Figure 4: Engine influence factors to be considered when operationg with plant oils

These results refer to the exhaust gas emissions, but this is only a tiny fraction of the numerous action fields of the fuel in relation to the engine. Figure 4 gives an overview of all the places in the engine where the fuel can have a positive or negative effect.

The whole situation is rendered even more difficult because the various engine makes and types can respond very differently. Apart from this, it is also necessary to take into consideration factors outside the engine or, better said, factors relevant before the fuel is consumed in the engine, for example long-term storage, danger class (flashpoint as high as possible), water toxicity, biological decomposability, etc..

Secondary effects could also be of importance. The fact for instance that the mass of soot particles in RME-exhaust gas is considerably reduced but the particles can eventually be larger than with gas oil [10] could be responsible on the one hand for reduced cylinder wear and, on the other hand, become relevant with respect to carcinogenic effects (access to the human lungs) and filter retention.

These aspects are only a small indication of the real complexity of interactions and often opposing trends of the various factors. The situation is enormously complex and by no means all factors which have a significant influence are known as yet.

General possibilities for development

The general possibilities and relationships which could be of interest for and have an effect on raw as well as transesterified plant oil fuels are [24]:

Primarily, the following parameters are selectable and modifiable:

- Chain length of fatty acids
- Degree of saturation of fatty acids

Relationships:

- The shorter the chains are
  - the greater the oxygen content of the molecules, thus less emission
  - the lower the CFPP (cold filter plugging point), i.e. the better the suitability for winter operation
  - the smaller the carbon content, i.e. the poorer the calorific value and therefore the smaller the power
  - the higher the cetane number (ignition capability).
- The more saturated the carbon chains are:
  - the better the chemical stability, the smaller the susceptibility of the fuel to oxidation and polymerisation
  - the higher the CFPP, i.e. the poorer the suitability for Winter operation.

The weighting of these factors can be different for raw plant oils on the one hand and transesterified plant oil fuels on the other hand, e.g. a (still) higher viscosity or a higher CFPP hardly plays a significant role in practice if it is necessary anyway to preheat the fuel.

Conceivable chemical modifications of the fatty acid chain [29] which, for example, increase the oxygen fraction or reduce the iodine number, are not our concern here. In any case, they would require a large effort for implementation.

Natural state raw rape seed oil fuel („ROR")

First of all an overview of the advantages and disadvantages [2]:

Raw plant oil

Oil production on a small technical scale (cold pressing) or in large-scale technology using oil mills. Affected thereby: Fatty acid profiles, some fat chemistry parameters and fuel characteristics.

Advantages:

- Local production and utilisation (with cold pressing)
- Short transportation distances (with cold pressing)
- Slightly cheaper (only fuel)
- 11 % oxygen (less soot and fewer particles)

- No sulphur
- Good environmental compatibility as fuel
- Good boundary lubrication (less wear, less lubricating oil consumption, less emission of particles)
- Good biological decomposability (ground)
- Very low water toxicity

Disadvantages:

- Special engines required
- High viscosity (requires 2 fuel circuits or preheating)
- Cannot be inter-regionally standardised (but regional standardisation is possible)
- In some cases greater fuel consumption
- Not optimised for oxidation catalyst (P content)
- Poor ignitability
- Increased coking tendency
- Operation somewhat more expensive (total life costs)
- Environmental compatibility poorer in the operational aspect
- Sometimes produces unpleasant odours (CDI method)
- There may be problems with long-term storage

The fatty acid profile of present day „00" rape seed cultivations consists almost exclusively of five fatty acids [13] (explanation of the abbreviated designations: e.g. C 18:1 means 18 carbon atoms, 1 double bond).

	Average contribution (%)
Palmitic acid C 16:0	6
Stearic acid C 18:0	1
Oleic acid C 18:1	58
Linoleic acid C 18:2	24
Linolenic acid C 18:3	11

Other fatty acids such as capronic acid C 10:0 or lauric acid C 12:0 are present only in traces. Fatty acids which contain no double bonds are called saturated, whereas those with one or several double bonds are called mono or poly-unsaturated.

We now come to some keywords for the desirable future characteristics of natural state raw plant oil fuels. Apart from the general goals for agricultural production such as large yield per acre with little and simple fertiliser, herbicide and fungicide requirements, at present chiefly the following characteristics appear to be desirable for utilisation of the natural state raw (or partially refined) plant oil fuels as engine fuel, without considering feasibility at this stage. These desirable characteristics are partly the same as for transesterified plant oil fuels. Except for the existing emission measurements [21, 13], we are here not yet concerned with substantiated research results, but rather with specialist opinions.

A good orientation guide for the minimum requirements for practical utilisation is given by the standardisation proposals for natural state raw plant oils which are to be used as fuel [15]. However, both information complexes are no more than orientation aids on the basis of present day knowledge.

Desirable future characteristics:

- Reduction of the viscosity by a factor of about 10 (small improvements are of no interest).
- Low tendency for oxidation, i.e. as far as possible saturated fatty acids (low iodine number).
- Low tendency for polymerisation. As far as possible saturated fatty acids (low iodine number).
- High oxygen content, such as lauric acid C 12:0 with approx. 15 % oxygen (coconut oil or the latest irish rape breed „Laurical") [15]. Both with iodine number 10!
- Good winter performance (CFPP = cold filter plugging point). Unsaturated fatty acids here favourable. Saturated fatty acids are here very unfavourable. High significance for motor vehicles, but little significance for stationary equipment (preheating!).
- As few unsaponifiable fat accompanying substances as possible (winter performance, coking).
- Iodine number 120 g/100 g. Represents the fraction of double bonds. The higher it is, the greater is the tendency to produce deposits and resin (e.g. linseed oil).
- Low sulphur content; 0.03 % by weight.
- Low phosohorus content, at present 25 mg/kg. Among other factors, determined by the oil production [13], e.g. cold pressing.
- Improved ignition capability (cetane number), at present about 38. Among other means, can be improved considerably by oxidative „fat degeneration" (indicator: peroxide number) with at the same time increased oxygen content, but offset by increased viscosity, considerably greater tendency to produce resin and decomposition of the natural anti-oxidation agents (tocopherols) [14].
- Lowering of the boiling point and continuous boiling curve. Short carbon chains.
- Increased oxygen content and smaller iodine number, possibly also achievable by specific chemical modification of the fatty acid carbon chains.

These desired characteristics are primarily determined by engine operation considerations and only to a smaller extent by the targets set for emission improvements. The extent to which future stricter exhaust gas legislation for motor vehicle operation can be fulfilled therewith is still unclear. In my opinion engines for natural state raw plant oils in industrialised countries are better suited for stationary systems (with fuel preheating and exhaust gas post-treatment).

Conclusions:

From the viewpoint of engine operation:

Carbon chains as short as possible, saturated as far as possible, lowest possible acid content (< 1 %).

For stationary systems (with constant engine speed) also long-chain saturated fatty acids (greater calorific value!) [23].

From the viewpoint of emission reduction (among other purposes): Short carbon chains, saturated (cetane number, oxygen content, iodine number). Low

boiling point, i.e. preferably long carbon chains (white smoke). No sulphur and phosphorus (Oxycat).

Transesterified rape seed oil „Biodiesel" (rape seed oil methyl ester, RME)

First of all an overview of the advantages and disadvantages [2]:

Biodiesel

(According to the present standard PME = plant oil methyl ester, possibly in the future, when production also uses animal source waste fat, FAME = fatty acid methyl ester).

Advantages:

- Can be used in existing engines.
- Use in the latest economising serial production direct injection engines.
- Standardisable.
- Normal viscosity (similar to diesel fuel)
- 11 % oxygen content (no soot, fewer particles produced)
- Unification of the physical and chemical natural oil properties of various kinds of oil plants.
- Very good ignitability.
- Good mixing capability with diesel fuel
- Little tendency for coking.
- Good boundary lubrication capability (anti-friction additive, little wear, low lubricating oil consumption, little particle emission).
- Optimum for Oxycat (no S, no P)
- Probably advantages for turbo-supercharging (acceleration), exhaust gas feedback, modern high pressure fuel injection systems (reduced NO_x production).
- Considerably reduced mutagenic activity (indication of reduced carcinogenic effect).
- Partly larger particle dimensions, i.e. better exhaust gas filtering capability.
- Good biological decomposability (ground).
- Very low water toxicity
- Great development potential for further fuel and engine optimisation.
- Joint utilisation of waste food oils and waste animal fat („multi feedstock")

Disadvantages:

- Considerable investments for transesterification systems
- Longer transportation distances.
- (Weak) aggressivity against some (substitutable) soft materials (gaskets, lacquers).
- Low load lubricating oil dilution for some engine makes.
- Increased amounts of ozone precursor substances could be produced compared with diesel fuel.
- Cytotoxicity (skin irritation) possibly slightly increased.
- Without oxycat, sometimes obnoxious odours.

– Possibly long-term storage problems for diesel fuel mixtures.

It is very fortunate that raw plant oil can be converted to a very diesel compatible fuel by a simple process requiring little energy, namely the so-called alcoholysis or transesterification. Thereby the fatty acids originally bound (esterified) to the triple bound alcohol glycerine are transesterified to single chain esters by replacing the glycerine with single bound alcohols (preferably methanol, but also ethanol).

The resulting plant oil methyl ester PME, „Biodiesel" is more diesel compatible than raw oil in almost all characteristics - from the viewpoint of engine running and of emissions. A preliminary standard already exists for PME (DIN V 51 606). The extensive work at present being carried out to draw-up the final version of the standard specification [17] and for quality control [18, 19] is a good basis for further considerations. More than 600 refuelling stations for Biodiesel already exist in Germany. Figure 5 shows an example of the extremely positive effect of transesterification [1].

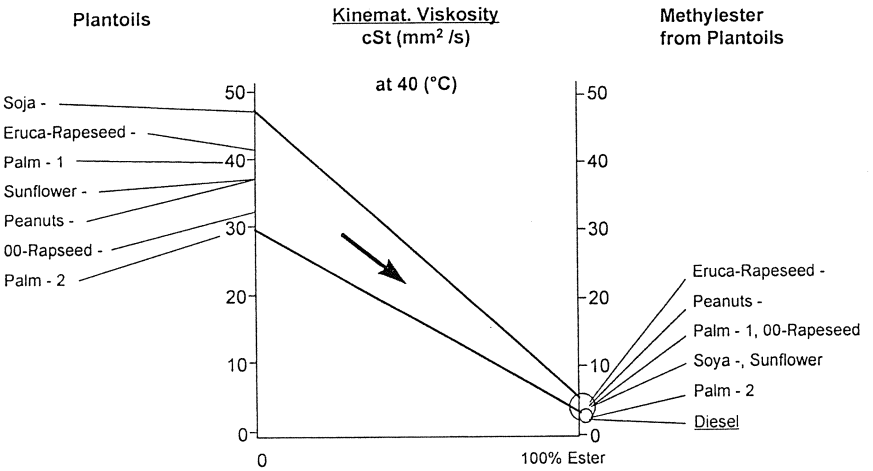

Figure 5. Viscosity improvement and unification from plant oils by esterification

It is here particularly remarkable that transesterification has not only „by chance" reduced the viscosity to approximately that of Petro diesel fuel - an important condition for mixing capability and interchangeability with the latter - but also that nearly the same viscosity is obtained starting out from very different kinds of raw oils, largely independent of the raw material properties, so that unification is achieved to a large extent.

Biodiesel **mixed fuels** are mainly additions to gas oil (e.g. 1,5 % or 30 %). They are of considerable significance. Here, to a certain extent, there appears to be an overproportional positive effect. Of course, this is of very great interest if it can

be confirmed. So far there are hardly any technical scientific publications on this subject.

Although all existing diesel engines (ranging from old ones right to the most recent new developments) can run on pure Biodiesel and, in general, thereby give better performance with respect to emission and operation than with Petrodiesel, there are nevertheless two lines along which these already very good results can be further improved:

- Optimisation for Biodiesel of the engines so far developed exclusively for Petrodiesel. (In contrast to the special engines already developed for natural state raw plant oils, comparable developments for Biodiesel do not exist as yet.)
- Further development of Biodiesel fuel for optimum diesel operation.

For the acute situation, the following points must be taken into consideration for the production of high quality Biodiesel fuel [22, 24].

- The cetane number increases for PME from C 22:3 to C 16:0.
- the melting point drops strongly from C 16:0 to C 18:3.
- The tendency for polymerisation and coking increases significantly with the iodine number, therefore an upper limit might have to be set at IN 115.
- The melting point of the mixture is lowered considerably if the saturated fatty acid esters can be removed (e.g. RME with CFPP = -36°C, but with IN 125).
- Biologically decomposable additives depress the melting point by about 10°C.

As already mentioned, future development of Biodiesel can be along various lines [2]: By plant breeding [20], by gene technology, by plant selection, by production, process control, blending, choice of materials used, post-treatment, additives, etc.

As sensible procedure, all these factors should be investigated in an iterative process of mutual progression and adjustment, i.e. as mutual optimisation of engine and fuel. This is an enormous research and development task for decades; I would even venture to say, for a century!

What initial steps are (already) now discernible for Biodiesel? Some keywords with respect to:

Desirable future characteristics:

- Reduction of polymerisation and oxidation tendency (degree of saturation!).
- Reduction of the iodine number (breeding, e.g. sunflower oil methyl ester, iodine number 135 - or 98 for high oleic sunflower oil methyl ester). Chemical modification of the fatty acid carbon chains.
- Reduction (or at least limitation) of the viscosity.
- Higher oleic acid content.
- Lowering of the boiling point. By incorporating shorter fatty acids (breeding, employment of other oils). Increased oxygen content, improved mixture production, less dilution of lubricating oil.
- Improved ignition capability (cetane number).
  - Short carbon chains.
  - Feed stock materials utilised (tallow?).

- Ethanol as transesterification alcohol. REE has significantly greater cetane number. 100 % composed of biomass (more difficult with regard to process engineering, more expensive).

– Increase of the oxygen content (but reduces the energy content!).
– Improvement of the winter performance (CFPP). (Short chains, additives or fractional distillation or crystalisation).
– Further improvement or at least retention of the excellent boundary lubrication capability.

Conclusions:

The procedure for further development of Biodiesel is - in some respects - rather different than that for natural state plant oil fuels.

Reasons:

– Viscosity limitation to gas oil values possible without preheating - and therefore necessary!
– The CFPP - without preheating! - must be suitable for winter operation too.
– The cetane number is good, but further increase is possible and therefore desirable - but this can, if appropriate, be considered only after attention to matters of higher priority.
– On the whole more engine-friendly.

Thus the short-term goal with respect to engine performance and a first cautious appraisal of emission.

Winter: Optimum is pure oleic acid C 18:1, or C 18:1 and C 18:2 mixture.

Summer: Fraction of short chain saturated fatty acids as large as possible.

All these numerous and multiply linked possibilities indicate the need for an enormous research effort whose full scope is not yet assessable, but they also imply great development chances and therewith an important contribution towards securing our future. We all know that an environmentally compatible transport system for our future mobile society is indispensable.

References

1. O. Syassen, Chancen und Problematik nachwachsender Kraftstoffe, *MTZ Motortechnische Zeitschrift*, 11 + 12 (1992).
2. O. Syassen, Biodiesel - ein vernünftiger Kraftstoff?, *Technische Überwachung TÜ* 1/2 + 3 (1996).
3. Symposium „Dieselmotorentechnik", Technische Akademie Esslingen, Conference Volume 1995.
4. R. Diesel, *Theorie und Konstruktion eines rationellen Wärmemotors*, Springer, Berlin 1893. Reprint VDI-Verlag, Düsseldorf 1986.
5. A. Pungs *et al.* Untersuchungen zur innermotorischen Rußoxidation unter dieselmotorischen Bedingungen, *MTZ* 11 (1996).
6. F. Pischinger *et al., Soot Formation and Oxidation in Diesel Engines* (Springer Verlag 1994).

7. H. Richter, in *C.A.R.M.E.N.-Conference Volume*, Würzburg 1994.
8. N.N., Technische Unterlagen, Motordaten AMS, Antrieb- und Maschinentechnik GmbH.. Schönebeck 1994.
9. H.-J. Kampmann, „Optimaler Kraftstoff für Motoren mit naturbelassenem Pflanzenöl." Telefax TMW, Thüringer Motorenwerk Nordhausen GmbH 11/1996.
10. J. Krahl, Bestimmung der Schadstoffemissionen von landwirtschaftlichen Schleppern beim Betrieb mit Rapsölmethylester im Vergleich zu Dieselkraftstoff. *VDI-Fortschrittsberichte* Reihe 15, Nr. 110, Düsseldorf 1993.
11. H. Fiedler, Messergebnisse und persönliche Mitteilung, FMC, Kiel 1996.
12. K. Maurer: Emissionsmessungen von nicht limitierten Schadstoffen bei Betrieb mit DK, RME und Tessol. Landesanstalt für landw. Maschinen- und Bauwesen, Uni Hohenheim und TÜV Bayern, 1993.
13. B. Widmann, „Verfahrenstechnische Maßnahmen zur Minderung des Phosphorgehaltes von Rapsöl bei der Gewinnung in dezentralen Anlagen". Dissertation. Techn. Univ. München. Institut für Landtechnik. Freising-Weihenstephan, 1994.
14. B. Widmann *et al., Verwendung von Rapsöl zu Motorentreibstoff und als Heizölersatz in technischer und umweltbezogener Hinsicht.* Volume 40. Techn. Univ. München, Bayerische Landesanstalt für Landtechnik, Freising-Weihenstepahn, 1992.
15. W. Körbitz, *Utilization of Brassica Oil as Biodiesel Fuel.* Brassica Oilseeds CAB International, Wallington Fond, Cambridge, UK, 353-371, 1996.
16. N.N., Protokoll zur 2. Nachbesprechung des 3. Arbeitskreises „Dezentrale Pflanzenölgewinnung." Techn. Univ. München, Bayer. Landesanstalt für Landtechnik, Freising-Weihenstephan 1996.
17. NN., „Normung von Pflanzenölmethylester (PME)". Arbeitsgruppe Biodiesel des AA 632. Ergebnisse der Einspruchssitzung vom 30.03.1995.
18. M. Wörgetter and H. Prankl, *Influence of the Jodine Number of Biodiesel to the Engine Performance.* (BLT Wieselburg). Third Liquid Fuel Conference, Nashville, Tennessee 1996.
19. N.N., 2nd European Motor Biofuels Forum. Conference volume, Graz 1996.
20. M. Mittelbach, *Der künftige Biodiesel, Möglichkeiten und Perspektiven.* Letter. Karl-Franzens-Universität, Graz 1996.
21. H. May, Neuere Untersuchungen über die Umweltverträglichkeit und die Dauerstandfestigkeit von Vorkammer- und direkteinspritzenden Dieselmotoren bei Betrieb mit Rapsöl- und Rapsölmethylester. *VDI-Berichte* Nr. 1020, Düsseldorf 1992.
22. J. Connemann, *Ölsaaten und deren weitere Entwicklung*, Tabelle12.96., Leer 1996.
23. H.-J. Kampmann, *Optimaler Kraftstoff für Motoren mit naturbelassenem Pflanzenöl.* Fax 11.96., Nordhausen, Harz, 1996.
24. W. Körbitz, *Zusammenstellung Biodiesel* (unpublished), 12.96, Wien, 1996.
25. H. May *et al.*, Vergleichende Emissionsuntersuchungen beim Betrieb verschiedener Dieselmotoren mit Dieselkraftstoff und Rapsölmethylester. *MTZ* 1 (1997).
26. M. Eberle *et al.*, Zum Einfluß der gekühlten AGR auf das Emissions- und Verbrauchsverhalten von PKW-DI-Dieselmotoren mit Common-Rail Einspritzung. ETH Zürich, *MTZ* 1 (1997).

Exhaust Components of Biofuels Under Real World Engine Conditions

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Introduction

The University Graz and also the Technical University Graz are both occupied with the chemical side of production as well as with the testing of Biodiesel under the most authentic practical service conditions [1,2,3,4]. From the experiences and results thus gained, two questions posed should be especially singled out here:

- What does the emission behaviour with Biodiesel look like compared to fossil diesel operation?
- Which fuel characteristics would we wish for, from a motorial point of view, in order to improve the operational- or emission behaviour of engines?

In order to at least begin to discuss the influence of different fuel characteristics to motorial behaviour a triple-comparison between diesel, rapeseed methyl ester (RME) and used frying oil methylester (UFO-ME) operation will subsequently be conducted.

Characteristic fuel properties

Now, before I go into the individual fuel-specific data I would like to briefly describe the production of UFO-ME.

The transesterification of rapeseed- and used frying-oil occurs practically in the same plant. UFO-ME is produced from used salad-oil and used nutrient fats, products that accumulate in every household and especially in large-scale catering establishments. After being collected, the used oil is warmed in order to separate the water and purified by filtering out food residues and other solid impurities. By normal customary use and proper storage no further purification steps are necessary before the actual transesterification with methanol and caustic soda as catalyst.

It can be deduced from table 1 that as well as UFO-ME, RME also features about a 15 % lower calorific value in comparison with diesel. This can be traced back to the correspondingly lower proportion of carbon in the fuel that is replaced by the 10 % oxygen deposit. This deposited proportion of oxygen is mainly

responsible for the positively altered emission behaviour in comparison to diesel operation.

Table 1. Properties of Diesel, UFO-ME, RME

	Unit	UFO-ME	RME	Diesel
Calorific value	kJ/kg	36500	37000	42700
Density (15 °C)	g/cm3	0.88	0.878	0.835
Cetane number		49	48	50
Stoichiometric A/F	kg/kg	~12.8	12.82	14.4
Viscosity (20 °C)	cSt	7.05	7.07	6.65
Viscosity (40 °C)	cSt	4.25	4.35	3.9
Flash point	°C	110	111	81
IBP	°C	307	~320	~180
EP	°C	340	~360	~350
CFPP	°C	-5	-12	+5....-24
Carbon	% (mass)	77.4	77.5	87
Hydrogen	% (mass)	12	12.1	13
Oxygen	% (mass)	10.6	10.4	0
Sulfur	% (mass)	<<0.01	0.0002	0.04

Additionally up front, a basic illustration that show the influence of the oxygen content on a very essential emission component of diesel engines, namely the exhaust opacity (figure 1).

In this illustration the decline in exhaust opacity is layed out above the oxygen content in fuel. The area around 10 % corresponds with the biogenic fuel and as we will later see, precisely this 60 % reduction in soot emission is also actually achieved in the engine.

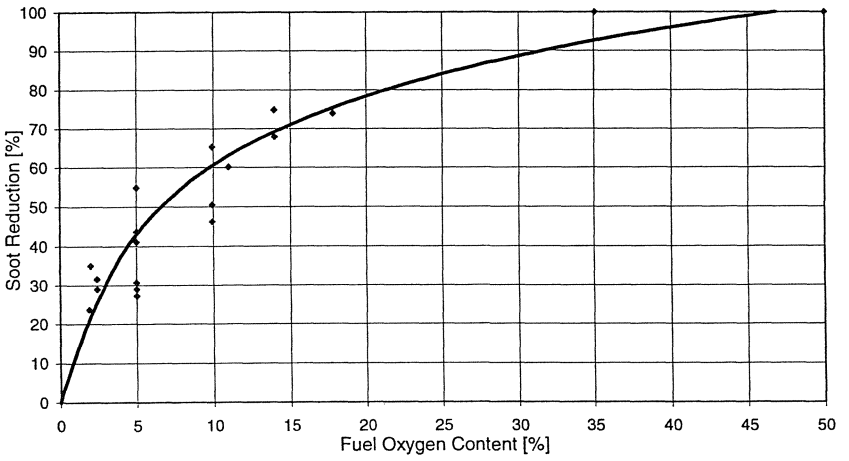

Figure 1. Soot reduction due to fuel oxygen content [5]

That means that the diesel fuel bonded chemically with oxygen indeed features a lower calorific value, but from the point of view of emission behaviour, brings quite decisive advantages with it. Two further essential differences occur in low-temperature behaviour and in the distillation range. Without additives, UFO-ME already begins to solidify between +5 and -5°C depending upon the composition of the used frying oil. The application capability of RME lies by about -12°C. If one could alter the chemical structure of the fuel in such a way that for example, through targeted intervention in the degree of saturation of the fatty acid molecules, then in practical service, without additives, considerable alleviation would be possible.

A fuel specification that has a strong effect in real world engine operation and especially in the partial load is the distillation behaviour (figure 2). Whilst diesel features the well known distillation range, the boiling points of RME and UFO-ME lie by 360 °C and 330 °C respectively. This has an affect not only on the lubrication oil but also quite massively on the particulate emission.

Here, without deliberation as to whether this is breed-technically possible or not, for example via the chain length of the molecules, a significantly lower boiling condition would be advantageous.

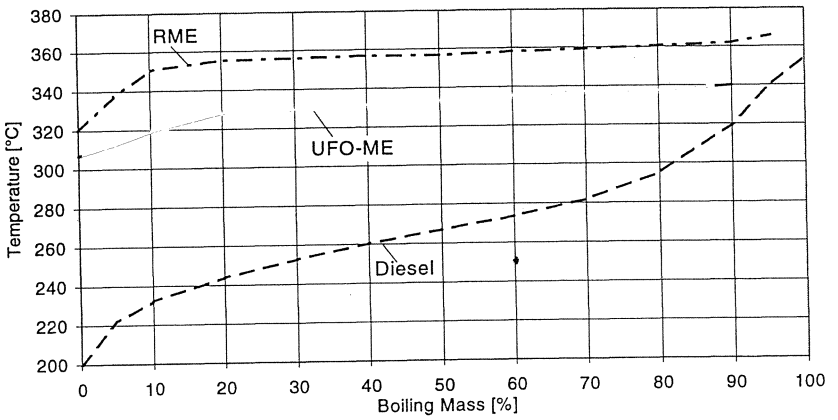

Figure 2. Boiling range of Diesel, UFO-ME and RME

Motorial results

The following test results are showing the emission reduction potential of biofuels, especially in combination with oxidation catalytic converter. The fuels are tested both in field tests and in test bed tests. In order to get an easy-to-survey basis for comparison, the following fuels are used for the tests at the test beds:

- commercial diesel fuel (0.04 % sulphur)

- standardised RME [6]
- UFO-ME of a quality corresponding to the preliminary Austrian standards for fatty acid methylesters [7].

In the framework of this project about 500 vehicles are looked after and selected vehicles and engines tested, repeatedly to some extent, on engine- and chassis dynamometers. The engines and vehicles were selected to cover the widest possible range of diesel engines used, thereby also permitting an analysis of the influence of the engine design or development stage. These vehicles and engines are

- two city buses,
- garbage truck,
- two tractor engines,
- heavy duty engine and
- a passenger car.

In addition, the vehicles operated in long-term tests with UFO-ME only were repeatedly tested. Each engine was tested both with and without oxidation catalytic converter.

The results of the individual engines are not presented and discussed separately but are visualised jointly in the form of bar charts. The presentation of the findings employs diesel with a 0.04 % sulphur content as absolute reference basis. Improvements or deteriorations related to the respective fuel or caused by the oxidation catalytic converter are directly reflected in the bar length.

Output and efficiency

These stationary and dynamic measurements have confirmed that the engine efficiency remains the same within the measuring margin, irrespective of the engine design and fuel used, with or without catalytic converters.

Carbon monoxide

Carbon monoxide is a non-critical emission component in diesel engines. Due to the oxygen combined in the case of UFO-ME and RME, the CO emission level was reduced by approx. 10 to 20 % both in stationary and dynamic operation irrespective of the engine design (figure 3). In all operational conditions, the oxidation catalytic converter causes a marked CO reduction, resulting in approx. 20-30 % of the original emission levels for all three fuels.

Hydrocarbons

While hydrocarbons are characterised by a more pronounced scatter of measured values, it may be said in general that a reduction by approx. 20-50 % of HC values was observed with RME and UFO-ME as compared to diesel fuel. This applies both to stationary and dynamic operation (figure 4).

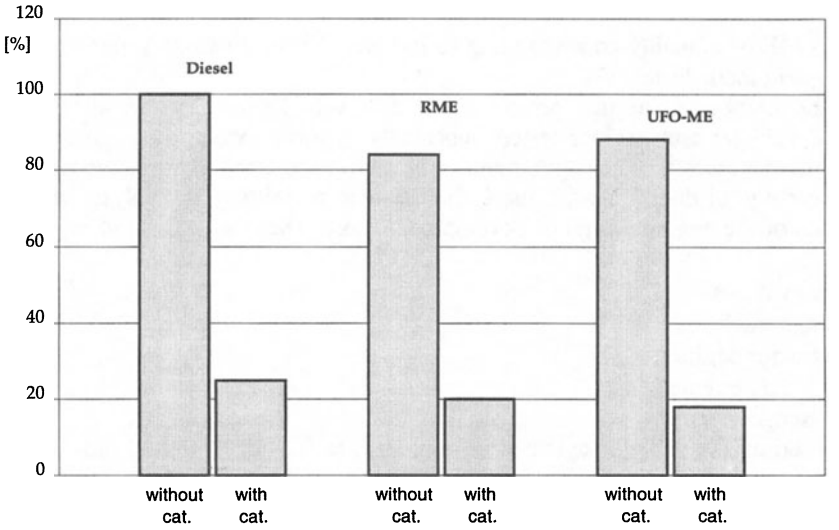

Figure 3. Comparison of CO-emissions

The markedly lower gaseous HC emissions of UFO-ME and RME are primarily caused by the higher boiling range of the fuels. As will be shown later in connection with particulate behaviour, the incomplete combustion of the HC compounds causes them to settle down on the soot, thereby reducing the gaseous component. Thus, in comparison to diesel behaviour, a shift from gaseous HC emissions to particulates takes place.

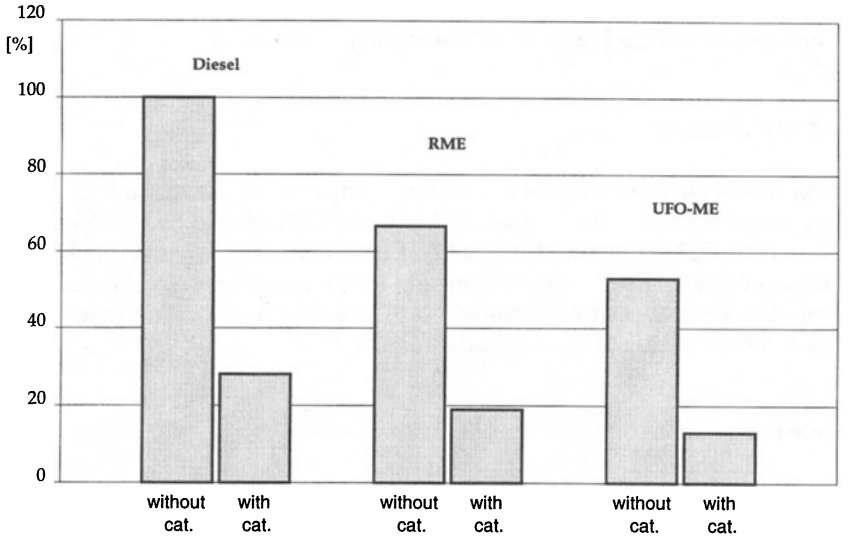

Figure 4. Comparison of HC-emissions

The oxidation catalytic converter is characterised by a conversion rate of 70 % in all 3 fuels. This means that HC emissions can be reduced to approx. 20 % of the reference level (i.e. diesel fuel without oxidation catalytic converter), if UFO-ME or RME is used.

Nitrogen oxides

While no distinction between stationary and dynamic engine load was necessary in the case of the parameters discussed above, i.e. output, consumption, CO and HC emission, this influencing factor is certainly significant with respect to nitrogen oxides.

In stationary operation, all three engines analysed presented the well-known NO_x increase of approx. 15 % as compared to diesel if RME or UFO-ME were used (figure 5). Dynamic operation of city buses, garbage truck or passenger cars on the roller dynamometer using both RME and UFO-ME as fuels did not result in any comparable NO_x increase (figure 6).

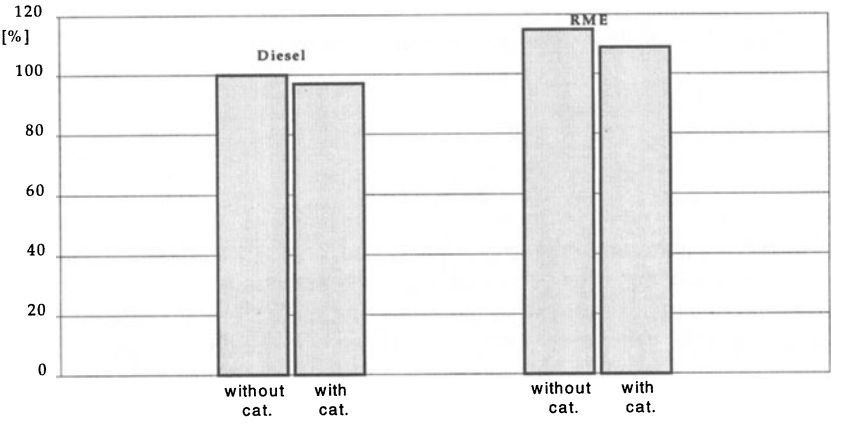

Figure 5. Comparison of No_x-emissions in stationary operation

The low soot percentage in the exhaust gases produced in RME and UFO-ME operation permits greater freedom for presetting the begin of fuel feeding. With both fuels, it is possible to set the moment of fuel feeding at several crank angle degrees later without incurring significant soot-related disadvantages. Setting the feed beginning at 5° later - a procedure that in diesel operation causes inadmissibly high soot levels - results only in a small increase of particulate emissions in biofuel operation but entails a 25 % decrease of NO_x (figure 6) values due to the lower gas temperatures.

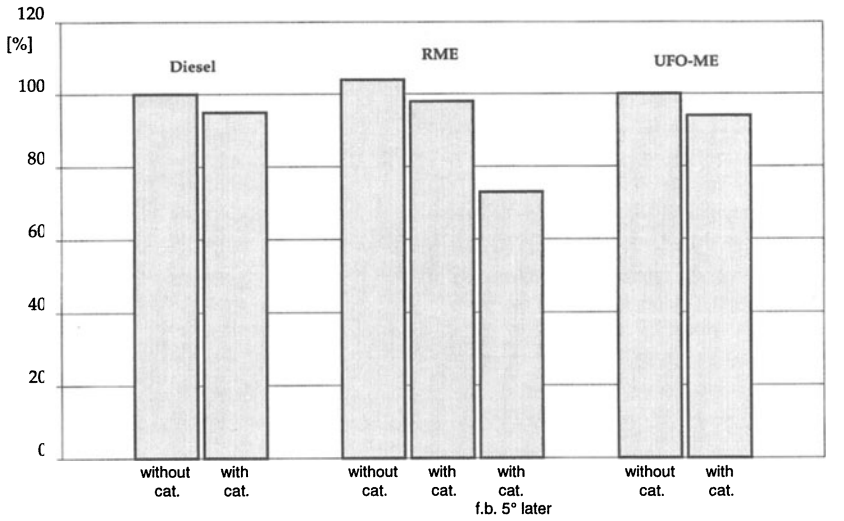

Figure 6. Comparison of NO_x-emissions in dynamic operation

Soot

Due to the oxygen combined in UFO-ME and RME, the advantage of both fuels lies in their soot emission behaviour, i.e. in exhaust-gas opacity.

Figure 7 proves this for an extract of the testing cycle of a city bus, in which exhaust-gas opacity values are listed for all three fuels. In particular in the acceleration phases, i.e. under high load, the opacity of the diesel exhaust gases is up to three times as high.

This is valid for older engines as well as very modern ones. Figure 8 shows the Bosch Smoke Unit for a 200 kW EURO-II heavy duty engine in the ECE-R 49 13 mode cycle. If the smoke units for diesel are already very low then the engine can be operated practically soot-free with Biodiesel.

Particulates

Now before I go into this emission component, a short note concerning the difference between soot and particulate emission. While one understands soot to be elementary carbon that developes exclusively in combustion, the particulate emission contains additionally other organically soluble components, in other words fuel that is not completely burnt up and lubrication oil as well as other solid or fluid exhaust components.

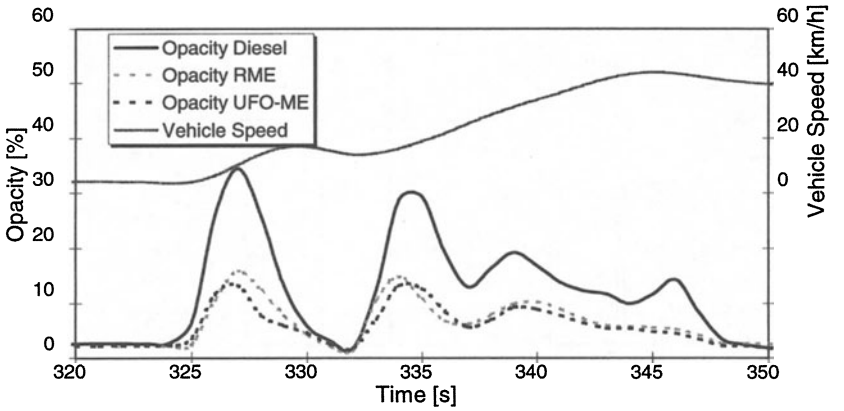

Figure 7. Comparison of opacity in the city bus cycle

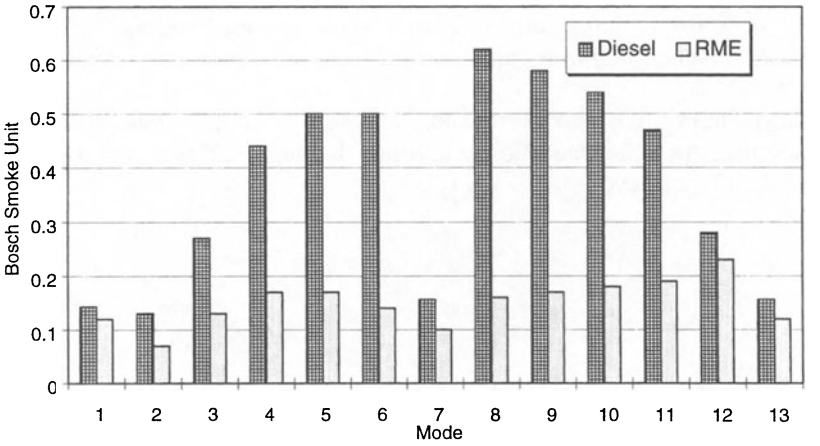

Figure 8. Comparison of smoke number in the ECE 13 mode cycle

Steady state operation

In spite of this clear advantage in soot emission and, consequently, carbon emission as well, the total particulate level is 20 to 70 % higher than in diesel operation, in particular in the stationary 13-mode cycle, which behaviour is strongly dependent on the engine used (figure 9). The reasons for this increase and the highly engine-dependent deviations are attributable to the higher percentage of organically soluble components with alternative fuels, i.e. to the incomplete combustion of the fuel contained in the exhaust gas.

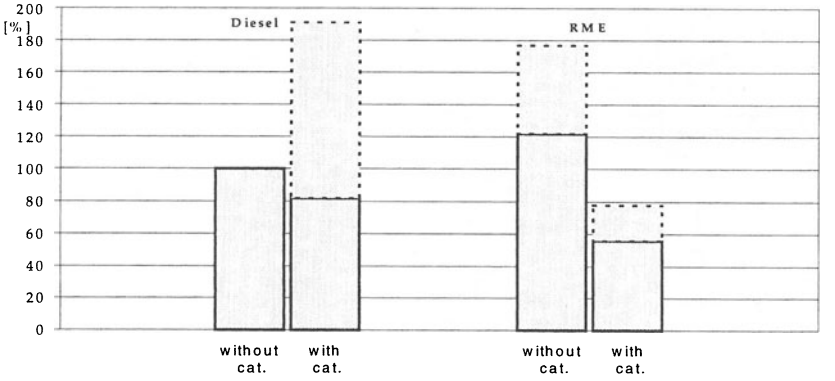

Figure 9. Comparison of particulates in stationary operation

Especially with older engines the soluble component percentage increases extremely with decreasing loads, in particular at low-load points, i.e. with relatively short combustion times and low mean temperatures in the combustion chamber.

The employment of catalytic converters leads to absolute contrary tendencies due to the differing fuel characteristics concerning boiling condition and sulphur content in the 13 mode cycle.

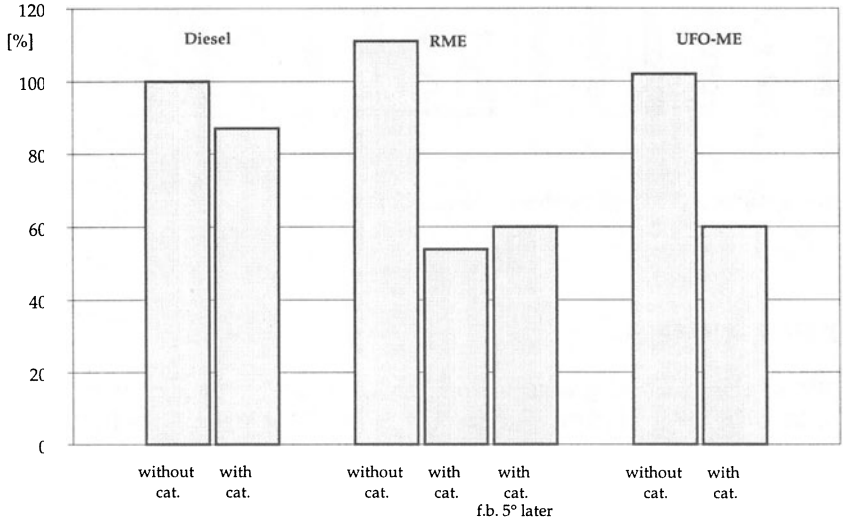

Figure 10. Comparison of particulate emissions in dynamic operation

Dynamic operation

In dynamic driving, conditions are altogether more favourable in terms of particulate emissions. Obviously, the engine does not cool down to such an extent as it is the case with lower load points in stationary tests with the effect that particulate emissions without exhaust gas after treatment in real world driving conditions are about as high as with diesel.

The use of the oxidation catalytic converter in the city bus operated in real road conditions, similar to stationary test operation with RME and UFO-ME, entailed a particulate reduction of 40 % as compared to diesel operation without oxidation catalytic converter (figure 10).

In addition, figure 10 describes the particulate emission behaviour for RME for a crank angle delayed fuel injection by 5°. It is known that this engine setting resulted in a 25 % nitrogen oxide reduction.

Polycyclic aromatic hydrocarbon (PAH)

The sum of the emission values for the urban city bus of 9 PAH components and also the benzpyrenes that are valid as a guide substance are layed out in figure 11. The results can thereby be thus summarised: In contrast to several other publications we have not determined any advantages for RME in the total PAH's. Solely the UFO-ME values lie significantly lower. The causes for such clear differences by both Biodiesel fuels is still unclear in spite of identical vehicles, test- and analysis procedures.

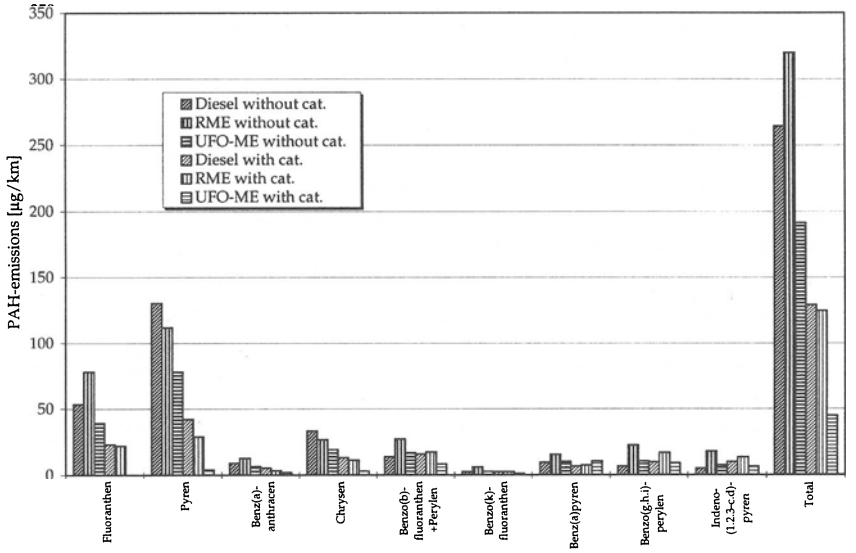

Figure 11. Comparison of PAH-emissions, dynamic operation

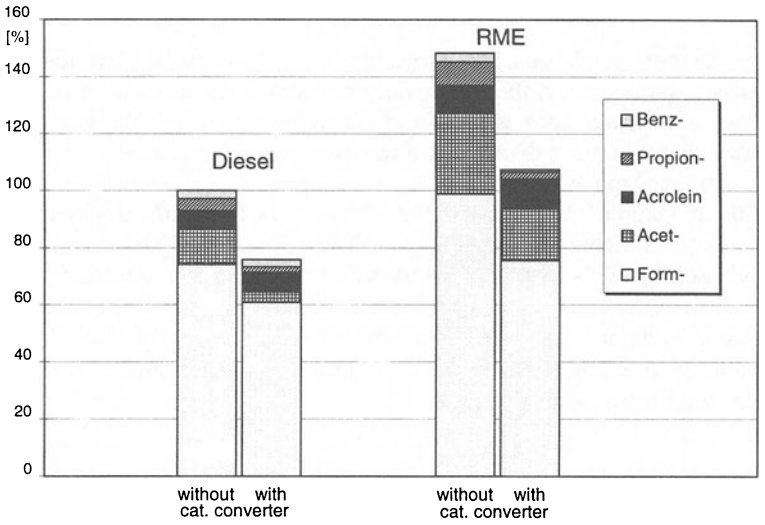

Figure 12. Comparison of aldehydes

Aldehydes

Aldehydes are measured to determine the possible causes of the typically different odour of exhaust gases of RME- and UFO-ME-fuelled engines. It has frequently been conjectured in relevant publications that this phenomenon might be caused by acroleine.

From the multitude of results it may be deduced that between stationary- and instationary operation, no systematic differences occur. Figure 12 shows a typical comparison between diesel and RME of individual aldehyde components. It is thereby noticeable that contrary to other differently opinionated literature details, the aldehyde emission with RME turns out to be higher than with diesel operation in spite of considerably lower total-HC-emissions. The main causes for this are the form- and the acetaldehyde. Interestingly, from these investigations it can not be concluded that acrolein represents the cause for the typical difference in aroma. Acrolein was found, at least in the same magnitude, by diesel as well as RME, with or without catalysator. Subjectively it must be clearly established that the employment of oxicate causes a considerable reduction in the intensity of the exhaust aroma.

Biodiesel and oxidation catalytic converters

As the above emission comparison shows, along with the reduction of the typical Biodiesel smell, the most important advantage of emission catalytic converters is the conversion of soluble particulate components, i.e. the conversion of incompletely burnt fuel.

The question is, for what driving performance are catalytic converters – usually a refitting solution for commercial vehicles – still effective. In order to answer this question, a range of vehicles of a fleet largely running on UFO-ME are to be refitted and subjected to constant monitoring. Two public service buses of the Grazer Verkehrsbetriebe have now completed a mileage of 120,000 km each.

Figure 13 shows the results for CO, HC and particulate emissions in comparison to raw emissions, i.e. without an oxicate. Although the emission reduction potential is lower than with optimised series applications due to the refitting status, the constant good conversion behaviour of all three exhaust components can be clearly seen across above the driving performance of 120,000 km. This means that Biodiesel enables to reduce exhaust with oxicate for vehicles with a state-of-the-art soot level permanently and effectively. If older or faulty vehicles are refitted that have a very high soot output anyway, there is a danger that the catalytic converter is displaced and thus loses its effect. According to our observations, this occurs more quickly with fossil diesel than with RME or UFO-ME due to the higher soot emissions.

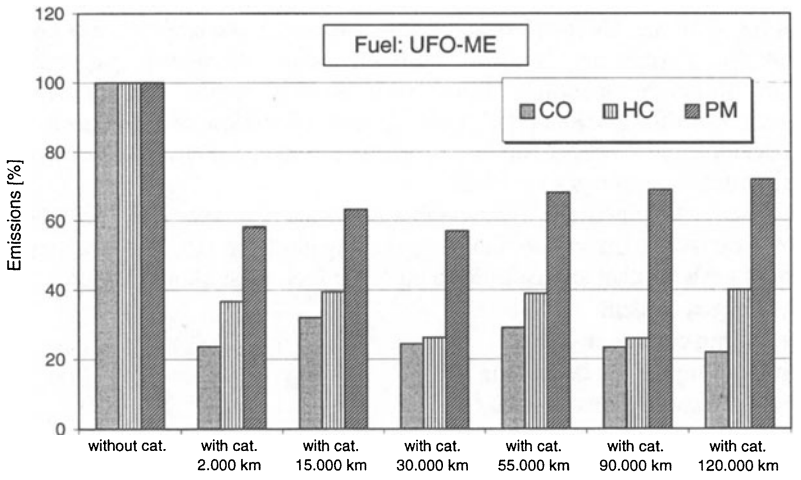

Figure 13. Influence of driven vehicle km on catalytic converter efficiency

Summary

In summary, it is valid, again with diesel without catalytic converter as a basis, that as well as RME, UFO-ME also displays slight to significant advantages with regard to CO- and HC-emissions without catalysator. The oxicate brings with it considerable emission reductions for all 3 fuels equally (see table 2).

Table 2. Comparison of emission behaviour

	Diesel		Rape-Oil-ME		Used-Frying-Oil-ME	
	without cat.	with cat.	without cat.	with cat.	without cat.	with cat.
CO	o	+ +	o +	+ +	o +	+ +
HC	o	+ +	+	+ +	+	+ +
NOx - steady state	o	o	-	-	-	-
NOx - transient	o	o	o	o	o	o
Soot	o	o	+ +	+ +	+ +	+ +
PM - steady state	o	- (-)	- (-)	+ +	- (-)	+ +
PM - transient	o	+	-	+ +	o	+ +
PAH	o	+	o	+	o	+

The nitrogen oxide-disadvantage noticeable by the stationary tests are no longer present under real world, dynamic operational conditions. Even clear NO_x-advantages for RME and UFO-ME resulted, possibly due to the late fuel injection.

This simplified summary becomes difficult when discussing particulate emission in stationary operation. Here, with modest engine load, massive disadvantages result for RME and UFO-ME in spite of modest soot emission. In real world dynamic employment conditions this is no longer the case, oxicate even produces significant advantages for RME.

We have not determined any considerable difference between the individual fuels by PAH-emission. Up to now, long-term testing has been very encouraging.

If we could wish for characteristics for a biogenic fuel, these should feature:

- A higher oxygen content
- A lower boiling condition
- A better low-temperature behaviour
- and a less intensive exhaust aroma.

References

1. T. Sams and J. Tieber, Use of Rape and Used Frying-Oil Methyl Ester under Real World Engine Operation, 5. Tagung „Der Arbeitsprozeß des Verbrennungsmotors", Sept. 1995, TU-Graz.

2. T. Sams, J. Tieber, M. Mittelbach, Biodiesel from used frying oil, Altener Symposium „Renewable Energy Entering the 21st Century", Sitges, Barcelona, Nov. 1996.
3. T. Sams and J. Tieber, Raps- und Altspeiseöl-Methylester im realen Fahrzeugbetrieb, 1st International Colloquium „Fuels", Esslingen, Jan. 1997.
4. M. Mittelbach, Production and Fuel Properties of Fatty Acid Methyl Ester from Used Frying Oil. Nashville, Tennessee. December 1992.
5. P. Tritthart, AVL-List Ges.m.b.H., Graz
6. ÖNORM C 1190. Kraftstoffe - Dieselmotoren, Rapsmethylester, Anforderungen. 1994.
7. ÖNORM C 1191. Kraftstoffe - Dieselmotoren, Fettsäuremethylester, Anforderungen. 1995.

Discussion of Session 1: Engines for Natural and Modified Plant Oil-Based Fuels

Presentation by **H Heinrich**: *The Automotive Industry's Views of the Standards for Plant Oil-Based Fuels.*

P Walzer: You referred to the DIN standard that has already been agreed upon for plant fuels. Although not finally decided, the industry agrees that it is necessary. I thought that rapeseed oil-based fuels fulfil the DIN already, and the remaining problem is that other plant fuels should fulfil the same standard. Is this correct?

H Heinrich: Yes. Our investigations of fuels on the market show that there is, in Germany at least, an adequate supply with no problems in fulfilling this norm. However, in Europe other countries with other plants, like sunflowers and olives, want these fuel crops included in the standard, too. Thus to achieve a broader base it has been decided, to make a plant oil ester norm, instead of one just for rapeseed oil methylester. From our standpoint we only need, for example, an iodine number of lower than 115. This must be achieved, no matter what kind of plant oil is used as raw material. For the fuel producers it is, of course, very important because with soy oil and some other plant oils there are problems reaching this low iodine number.

J Schell: You stressed much the fact that control is needed to ensure that the customer obtains a fuel corresponding to these norms. Will this add to the costs of plant methylester fuel?

H Heinrich: If handled carefully, control will be unnecessary. Sometimes however, filling stations have a lot of water in their tanks. If this water were to get into the engine, this would cause problems. Most filling stations have no problems with this.

G Röbbelen, plant breeder: You have given quality standards for biofuels and added that it might be possible to reduce the iodine number by hydrogenation. My question is: what potentials has optimising the plant oils with regard to the process of fuel production by changing e.g. their fatty acid composition by plant breeding?

J Connemann: I represent Oelmühle Leer Connemann GmbH & Co., affiliate of Oelmühle Hamburg AG resp. ADM Archer Daniels Midland Company, Decatur, III. (USA). We are the only large international Biodiesel producer,

producing currently 80,000 tons a year and expanding. We have no problems with the factors that have just been mentioned. We see no need for additional hydrogenation because, normally, rapeseed oil—at least in our European region—has an iodine number of 110–114, or even 116. Other oils, such as palm oil etc. could be added to lower the iodine number a little, but this is unnecessary at the moment. With regard to controls, we have quite a big, but rather sophisticated and very well controlled automated process, so there is no additional cause for further control.

Presentation by **A Schäfer**: *Vegetable Oil Fatty Acid Methyl Esters as Alternative Diesel Fuels for Commercial Vehicle Engines.*

J McDonald: Evidence from the United States suggests that oil-change intervals can be lengthened with Biodiesel fuel and the viscosity increase reduced or eliminated by using lubricating oils that have a higher degree of anti-oxidant additives. Have you any experience with trying to modify lubricating oil to increase the oil-change interval?

A Schäfer: We have a lot of experience with different lubricating oils and although our operating instructions allow the use of more than 1,000 oils, we cannot test all oils. We have dramatically bad experience with the one or the other oil type, e.g. with one typical Malaysian market oil that can be used with diesel fuel without any complaint. But we had dramatic engine sludging, forcing us to break off the test. However, if a good quality diesel engine oil is used, S-3 quality or higher in Germany, as defined by Regulation 228.3, there is no chance of sludging, at least as far as we know. However, given the increase in viscosity of plant oils, in the interest of our customers we must remain on the safe side and so we recommend that our commercial vehicle users reduce the oil-change interval by half. Passenger car users can retain the conventional oil-change interval because it is in any case shorter. To date we have not used a dedicated lubricating oil for the same reasons as with the dedicated engine. Who should develop it? I suspect that no company in the mineral oil industry is interested in developing a special Biodiesel oil, so we must use existing oils. Fleet owners, who have in part very old vehicles not to be modified for Biodiesel use, also normally won't want to have two different oils in their fleet and will wish to maintain their own oil. We thus use a certain quality level and recommend halving the oil-change interval.

R Töpfer: I would stress co-development of engine and plant oil. A symposium like this should initiate research into both aspects: development of special engines and securing improvements of an appropriate and extremely well adapted fuel for these and so obtain, in the long term, the advantages of biofuels. So again: what is planned in the long term with regard to the development of engines in relation to plant oils?

A Schäfer: As long as there is no chance of replacing more than 1 or 2 % of the fuel demand by vegetable oil ester, it is completely impossible to develop such dedicated engines, at least for companies in the global free market. As Dr.

Heinrich pointed out, this would be a completely new engine and the costs tremendous, sums of money that nobody today is willing to provide. If, some day, it were possible to replace 30 % or 50 % of diesel fuel consumption by vegetable oil esters, this will certainly be possible, but this is not going to be within the next twenty years. The other point is, if you have resources like natural gas, it is quite clear that a dedicated engine is necessary, because neither a commercial vehicle diesel engine nor other engines are able to burn natural gas as it is. Since the reserves are tremendous, it would make sense to develop a dedicated engine. These thoughts could be extended: some day, for example, the triglycerides could be replaced by waxes. Perhaps the fuel would then be better, with no glycerides and hence no bad smelling and irritating acrolein in the exhaust gas. Although this is not a vegetable oil, this could be the next step and, quite clearly, a lot could be done. However, with regard to engines to be sold today or in the next twenty years, there will be no dedicated engine.

Presentation by **O Syassen**: *Diesel Engine Technologies for Raw and Transesterified Plant Oils as Fuels: Desired Future Qualities of the Fuels.*

T Sams' presentation is given by **W Körbitz**: *Exhaust Components of Biofuels Under Real World Engine Conditions.*

P Walzer: As stated at the beginning, the purpose is to prepare a kind of shopping list of plant fuel properties that, in the eyes of the motor engineers, would enable plant fuels to contribute really importantly to transportation fuels. As a basis for the following discussion on biotechnological developments of the plants Table 1 contains some lead statements. Please feel free to correct and add to each statement.

Table 1. Targets for biotechnological/gene technological improvement of oil plants.

- Fulfil PME DIN 51606 with additional plant oil fuels
- Increase oil yield per ha by 100 %
- Reduced glycerol/liquid wax esters
- Reduced unsaturated fatty acids with 2 and 3 double bonds
- Fatty acid pattern for low cold filter plugging
- Shorter chains? (problem of smell, energy content) : More research!

The list has 3 main topics. First, it should be ensured that all usable plant oils, not only the rapeseed derivative, fulfil this standard. This allows their use in present engines and would achieve already better acceptance. Next, and possibly already a target for bio- and gene-technology, is that the oil yield from a given area should be increased greatly. Finally, attention should be paid to all the engine-related properties required to obtain improved combustion processes, less toxic emissions and lower particulates. To the first issue: should and can the PME standard be a target for all plant fuels?

M Frauen, Norddeutsche Pflanzenzucht: As a plant breeder I think it is important to consolidate production. It is important to have plants other than only

rapeseed, and genetic modification of the other plants will come also. But rapeseed is a good starting point and, for Central Europe, the major crop. On a global basis, however, other species, like sunflower, palm oil, soybean are important.

G Röbbelen, plant breeder: If there really is a diversity of plant oils available, then the consumer must think about adding additives or whatever. Nature cannot be changed just to fulfil the requirement of a diesel norm.

P Walzer: This leads to the question of specialised engines. This is not a realistic goal.

G Röbbelen: Why can we not regard a niche to be an opportunity for creating new special values?

A Schäfer: This is correct, Professor Röbbelen, if a special value really is created by using that fuel, and providing the same effect cannot be achieved more cheaply by using, for example, other fuels such as natural gas. For example, if a special low-emission vehicle is sought for downtown or underground usage or usage in specially threatened or non-containment areas in the United States. If the same effect can be achieved more cheaply, then a special value needs not to be added to that fuel. Secondly, I do not believe there will be additives able to convert all different vegetable oils into fuels suitable for existing engines. Is there an additive preventing linolenic acid from polymerisation? I do not think so. We thus have to adapt the fuel to the engine, because this will be the only chance to enter the market with this type of fuel. It will also have to be the existing engine, because otherwise there will be the "chicken-and-egg" situation, and nobody will be prepared to invest as the first. Thus the direction will be adapting the fuel to the requirements of existing engines, at least for the next 10 or 20 years.

P Walzer: Most of us will agree then, that not all fuels, but at least more than just rapeseed, should fulfil the DIN standard. The second issue is whether we feel that biotechnical means will be necessary to obtain a higher yield from those areas today available for these plants. As has been discussed, there is even doubt whether these areas can be expanded very much in the future, for political and other reasons. Given costs and efficiency, efforts will be necessary to obtain a higher yield. This in itself maybe a big task for biotechnology. Improvements of at least 30 % are necessary to make the plants more resistant.

A Schäfer: If a significant volume of production is really desired, the productivity factor, in terms of kilograms of oil, must increase by 100 %. This will be difficult, but this is the requirement.

K Thurau: Are the requirements for non-food oil the same as for the food oil?

H Bertram, Union for the Promotion of Plant Oils:. The stable state of the market has led to a reduction of Germany's arable land area by 5 % in recent

years, and for Europe it will be nearly 10 % in the next year. In Germany more than 1 Mio. ha arable land will have to be taken out of production, and probably more in the next 10 or 20 years. If agricultural underdevelopment shall be avoided, new markets will be necessary. Indeed, reducing arable land area is not a good perspective for entrepreneurial agrarian policy. This is why we believe that the whole topic of this meeting is innovative and directed towards the future of agriculture and is also a good basis for automotive engineering, for car production. Both Mercedes and BMW have contributed to innovative development and strategy by using these fuels in their engines. Given the trade situation, apart from a stable food market and increasing demand on the world market, nearly 20 to 30 % will have to be devoted to non-food production.

With respect to the quality aspect of your question: non-food and food usage of oils require different qualities in principle. This was more applicable in the past than it is today. For instance, the views of the margarine industry with respect to several features of the margarine are totally different today than they were in the past and in the USA, the question of the importance of certain polyenoic fatty acids was also answered more critically in the past than it is today. This means that we have to produce a totally different oil with a high oleic acid content, and if double bond components are not desirable for fuel production, then we can take advantage of the fact that plant oils with high oleic acid content today are highly desired in human nutrition, too. In other words, the plant breeding programme will be the same as for fuel development. It is not economic to produce rapeseed for Henkel and the automotive industry on the one hand, and a totally different rapeseed for the food markets. There must be one common production.

P Walzer: It is well understood this area is important, and that the target for yield improvement is 100 %. The third item was the list of properties that may help engineers improve combustion processes, lubrication and long endurance stability of today's engines.

J Connemann: For glycerides the DIN standard is today 0.25 %, our product has normally only 0.08 % and could be reduced to 0.04 %.

A Schäfer: It is not a question of the composition of the vegetable oil. Vegetable oil is by definition triglyceride, and therefore it can't simply be removed. The fuel producer must know how to take it out when transesterifying the oil into Biodiesel. This may not be influenced by plant breeding technologies, because the same amount of glycerol will be in the raw material in any case.

R Töpfer: I would like to comment on that. Eliminating the glycerides has no top priority at the moment. In the long term, if acreage is increased, the point may be reached where no further glycerine can be put onto the market. Glycerine is a by-product and contributes to cost stabilisation, too. If the amount of glycerol is increased, in the end there will be a problem in the price politics. In the long term liquid wax esters may be one way of replacing triglycerides in plants as a source of biofuels. This is a completely new development. Wax esters are being

investigated, although more in the direction of cosmetics, but they may be envisaged as biofuel, too.

P Walzer: The next topic is unsaturated fatty acids.

J Connemann: The aim is to reduce only that fraction of the fatty acid content containing two or three double bonds. These fatty acids are not totally unsaturated. Mono-unsaturated fatty acids are of interest, as are the dual-unsaturated, at least for nutrition, because a very valuable oil is sunflower seed oil, which has a high content of this type of fatty acids. Really disturbing is the triple-unsaturated fatty acid, which is predominant in linseed oil, for example.

P Walzer: The next topic is the fatty acid pattern for low cold filter plugging.

R Töpfer: To reduce the amount of fatty acids that cause cold filter plugging, it is necessary first to specify which fatty acids should be reduced. If the saturated long-chain fatty acids are to be reduced, they must be replaced with something. The question is, with what? Oleic acid or a short-chain fatty acid?

A Schäfer: I cannot evaluate the real importance of that because there are quite good flow improvers currently on the market. I am not quite sure if this type of additive will work in all those materials we could imagine as Biodiesels. If the problem can be solved with only a few hundred ppm of a cheap and biodegradable additive, so as not to lose the environmental advantage, then this should be done. If this is not possible, then the fatty acid pattern must be changed for low CFPP. Perhaps Dr Connemann could answer this question. Do you believe that the additive you are using today is suitable for all types of fatty acid? Take, for instance, an imported palm oil ester; do you believe you could handle that under our temperature conditions with the additive you use today?

J Connemann: No. The cold filter plugging point (CFPP) of rapeseed oil methyl ester without any additive is around -10 °C. With an additive developed together with Exxon, Paramins and others, we achieve regularly -22 °C which is quite good. From Wieselburg we know that freezing out the saturated fatty acids, the content of which in normal rapeseed oil is about 6–7 %, reduces the CFPP to -36 ° without any additive. However changing or eliminating by taking out these 6–7 % of the fatty acids increases the iodine number to 125, increasing the sensitivity against polymerisation, which is undesirable. The right balance has to be found.

G Röbbelen: This is very interesting because going into high oleic acid contents automatically reduces not only the already low amount of saturated fatty acids in rapeseed, but also the polyunsaturates. This is an area where nutritional and the non-food uses are on the same line. The plant breeders will certainly agree that this is a good direction.

H Prankl: I am from the Federal Institute of Agricultural Engineering in Wieselburg, Austria. We have developed a process technology for finding a new diesel RME with very cold temperature properties, below -30 °. The saturated C_{16} and C_{18} fatty acids could be reduced, but not be removed completely. Our need is for a new type of plant oil with a reduced content of C_{16} and C_{18} saturated fatty acids, to achieve better CFPP properties.

W Körbitz: I think the future of Biodiesel lies in a clever blend of various raw materials. This may be compared with the feed industry where, when a protein feed is produced, the farmer is simply guaranteed a certain protein content. Where the protein is obtained depends on whether there is cheap soy-bean available, or fish meal. In our case, the end product must be Biodiesel. The CFPP can be higher in Ireland, but it must be low in the Austrian Alps. Plant breeders and other fatty acid suppliers must consider what they can contribute in this regard. The double-low Canola already has a very low content of saturated oils, and in that respect it's by chance an ideal raw material. But this situation can be improved.

R Töpfer: A question to Dr Körbitz: Could you define a blend for methyl esters? If so, lists of the different types of different plant oils that would be needed to meet these requirements could be composed. I agree with Professor Röbbelen in that there will not be a large number of different varieties in large-scale production. There will probably be just a couple of different qualities grown, and from these the required blend will have to be made. The question is: what kinds of blends will be required for plant methyl esters?

W Körbitz: The first example is CFPP. Regardless of where one is, there are different requirements in summer and winter, as is the case for fossil diesel. More flexibility is possible in summer and oils with a higher saturation level can be used, regardless of origin, including, in theory, a small share of palm oil as well.

R Töpfer: This implies two, i.e. summer and winter, qualities and the necessity for mixing various types of plant oils to achieve the desired quality. Seed will have to be stored when the winter material is not needed and the spring material will be used immediately if the requirement is there. This raises the question of handling and management. The problem is to define the blends required for summer and winter, including the types of fatty acids—perhaps a spectrum of fatty acids will be necessary. The blend can then be defined according to the DIN norm and plant breeders can try to develop the desired oil quality to meet these requirements.

W Körbitz: Certainly there is a logistic challenge, as for fossil diesel. One has to switch in time and keep the logistic costs as low as possible.

P Walzer: The final topic is that of desired chain length.

A Schäfer: It has been mentioned that shorter chains are desirable. What emission measurements justify shorter chain lengths?

W Körbitz: Data of Dr Sams showed improvement in emissions with increasing oxygen content. I'm not aware of any trial in which Biodiesel has been tested with oxygen contents of 10, 12, 14 or 16.

J McDonald: One must be very careful with large increases in fuel oxygen content. Much work has been done in the USA on fuel oxygenates to meet some of the requirements of the Clean Air Act Ammendments with considerable research at the Department of Energy National Laboratories. One finding is that the response of diesel emissions to fuel oxygen content is nearly identical to the response of emissions to air oxygen enrichment. At Argonne National Laboratories it was found that a point of no return can be reached. If a certain degree of oxygen enrichment is exceeded, no possible injection timing retard will reduce oxides of nitrogen (or NO_x) emissions. In some cases oxides of nitrogen emissions were doubled or tripled. This would be a serious problem for urban areas with ground level ozone problems. So there is a point of no return, and hence a trade-off, at least for direct injection engines (not necessarily for indirect-injection engines), between oxides of nitrogen emissions and particulate emissions. The problem is clear: if one of the advantages of using Biodiesel is the reduction of particulate emissions, but injection timing must be severely retarded to achieve acceptable oxides of nitrogen emissions, there may be a point beyond which there is no net gain.

N Martini: It would be very helpful if you could define the point of no return with respect to the ratio of oxygen and carbon chain lengths.

J McDonald: From data published this year by the University of Michigan and Argonne National Laboratories comparing oxygen enrichment (in that case was air oxygen enrichment) with fuel oxygen enrichment, it might be possible to define a point of no return for fuel oxygen content, at least theoretically. It can also be done using engine modelling. KIVA engine modelling at the University of Michigan has been able to predict oxides of nitrogen emissions very closely. Thus it might not require an engine test programme, but just clever computer programing and a very clever researcher using the software.

A Schäfer: I would like to make one more remark on chain length. The difference in oxygen content between the shortest chain length vegetable oil, coconut oil, is about 14.5 % compared with almost 11 % for RME for example. That doesn't seem to be too great a difference. On the other hand, we have had very bad experience with an only 20 % blend of coconut oil-type ester with RME. Following the Fulda fleet test there were dramatic complaints from the public with the press reporting that the whole Fulda area smelt like a French fries restaurant. That product is not marketable. We had to refuel with conventional diesel fuel: garbage trucks, for example, where the workers stand outside and must breathe the exhaust gas. This was all due to the addition of coconut oil. Therefore I am really not sure that shorter chain lengths is a good way. I would recommend that this at least be tested before such feedstocks are bred.

N Martini: What about mixing oleic acid and shorter-chain fatty acids, e.g. C_8 and C_{10} fatty acids, to achieve partial increases in oxygen content?

J Connemann: Again, there may be the point of no return, but this will certainly be far higher than 12, 15 or 18 % or so because, for instance, with dimethyl ether, with an oxygen content of about 30 % these problems do not occur. With every increase in the oxygen content, heat and energy content is also lost, thus adding to costs. We are therefore not interested at all in increasing oxygen content. It would be better to lower it, but this is not possible because it depends on chain length and the short-chain fatty acids smell very bad, very sharp. We are thus not interested either in having extremely short chains.

J McDonald: Some of the combustion characteristics of dimethyl ether are fundamentally different, so a comparison just on fuel oxygen content cannot be made. Dimethyl ether (DME) has practically zero ignition delay in a diesel engine. And a fundamental characteristic - combustion knock - is practically non-existent, i.e. no premix burn. Oxides of nitrogen emissions from a fuel of that type really can't be compared with a fuel like Biodiesel that is more similar to diesel. (Note added in proof: DME also has no carbon-to-carbon bonds, thus cannot form soot by the most common mechanism: fuel pyrolysis. Therefore, the usual trade-off of exhaust gas recirculation rates and injection timing causing particulate matter and oxides of nitrogen emissions to go in opposite directions do not apply to DME.)

Session 2:
Toxicological and Environmental Aspects Related to the Use of Plant Oils as Fuels
(*Chairman: Günter Oberdörster*)

Toxicological and Environmental Aspects Related to the Use of Plant Oils as Fuels

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Introduction

Both in Europe and in the United States programs are underway to test the feasibility of using plant-derived oils as diesel fuels, either as full replacement (Europe) or as blend with petroleum-based diesel fuel (USA). This chapter will present and discuss data about toxicological and environmental aspects related to the use of Biodiesel. Due to the lack of a sufficient number of studies with Biodiesel addressing these issues, comparisons will be made with numerous data from petroleum-based diesel studies aimed at the toxicology and potential carcinogenicity of petroleum diesel exhaust. Individual presentations will compare available data about the mutagenic potential of diesel exhaust, discuss Biodiesel fuel constituents and their relationship to exhaust constituents, and finally, evaluate their ecotoxicological impact including biodegradability and effects on aquatic/marine life. These discussions will form the basis for answering a key question: Can we provide useful information to plant scientists about relationships between harmful products in the exhaust and their fuel precursors which will aid in the breeding of genetically altered oil- producing plants yielding improved Biodiesel fuel so that undesirable exhaust constituents are minimized?

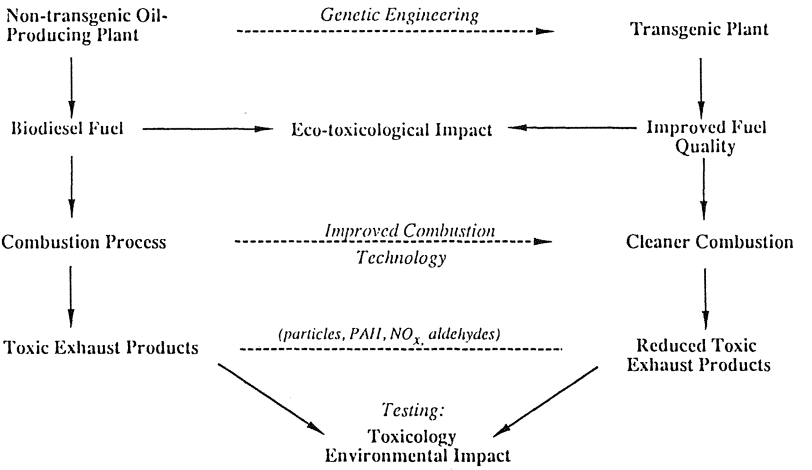
Although Biodiesel fuel and Biodiesel exhaust may offer advantages over petroleum diesel with respect to protection of human health and the environment, additional research is needed to characterize the potential toxicological and ecotoxicological impact. Knowing the most significant toxic constituents in diesel exhaust with respect to human and environmental toxicity opens the possibility of developing strategies aimed at altering specific biofuel precursor constituents via genetic engineering of plants such that exhaust toxicity will be reduced. Particles and particle-associated polyaromatic hydrocarbons (PAH) are among those compounds with a potential of significant toxicity. High concentrations of particulate and gaseous constituents of petroleum diesel exhaust, and by implication potentially also of Biodiesel exhaust, can be causally associated with adverse effects. For example, results from petroleum diesel research have shown that exhaust extract is mutagenic based on the PAH content. Furthermore, inhaled particles of petroleum diesel exhaust have been implicated as causative agents for lung tumor induction in laboratory rats after long-term exposure to high exhaust

concentrations as will be discussed in this chapter. Although the relevance of this finding for low exposures of humans to diesel exhaust is still debated, a decrease in particulate exhaust emissions is most desirable and would be in line with recent efforts by the U.S. EPA and regulatory agencies in Europe to reduce ambient levels of fine particles. Some of the results of human epidemiologic studies in cohorts with occupational exposure to petroleum diesel exhaust suggest a small increase in the risk of lung cancer. However, due to imprecise characterization of exposure and difficulty in adjusting for confounders such as smoking, the certainty of this elevated risk has been debated. Compared to studies in animals, the low particulate concentrations of diesel exhaust encountered both in occupational and urban environments are unlikely to induce lung tumors in humans by the mechanism of particle overload that is assumed to be the basis for the rat induced lung tumors.

Presently available data presented in this chapter indicate that the use of Biodiesel fuel may indeed result in less particulate emissions as well as lower PAH concentrations, although the PAH profile may be different. On the other hand, more NO_x emissions and higher concentrations of formaldehydes might occur, which makes it difficult to evaluate the overall toxicity of Biodiesel exhaust lacking appropriate toxicological studies. Thus, at present we are left with the uncertainty to base an evaluation of the potential toxicity and carcinogenicity of Biodiesel exhaust on extrapolations from results of petroleum diesel exhaust by comparing levels of toxicologically most significant constituents. However, since compounds derived from the respective combustion processes are basically similar, confidence in the predictions derived from the comparisons should be high.

With respect to the environmental impact from the use of Biodiesel fuels, it appears that improved biodegradability of the fuel and reduced greenhouse gas emissions in the exhaust through CO_2 recycling are advantageous compared to petroleum diesel fuel. The ultimate goal of the research on Biodiesel will be to reduce potential adverse environmental and toxicological impacts by improving both biofuel composition and combustion technology. Table 1 outlines schematically a multi-disciplinary approach to achieve this goal by using genetic engineering techniques for altering the oil-producing plants as well as mechanical engineering techniques improving the combustion process so that in appropriately designed toxicological and ecotoxicological studies the final product Biodiesel fuel as well as exhaust can be tested. This process requires collaborative efforts of toxicologists and ecotoxicologists with plant geneticists and automotive engineers. The contributions in this chapter will discuss this and related issues with the aim to identify undesirable constituents in biofuel and biofuel exhaust, and perhaps to propose further specific toxicological and ecotoxicological testing to fill existing knowledge gaps. However, performing these additional tests is only meaningful after the improvements outlined in table 1 have been implemented.

Table 1. Improving Biodiesel–fuel and –exhaust: A multidisciplinary effort



Goal: To reduce potential adverse environmental and toxicological impact of biodiesel-fuel and -exhaust by altering bio-fuel composition.

Health Issues Concerning Inhalation of Petroleum Diesel and Biodiesel Exhaust

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Abstract

The public and regulatory agencies will question the comparative health risks from inhaling Biodiesel (BDE) and petroleum diesel (PDE) exhaust. The principal health concern for inhaled PDE is lung cancer from the respirable soot, which contains known mutagens and carcinogens. Epidemiology suggests that heavy occupational exposures to PDE may slightly increase lung cancer risk; however, the magnitude of the risk is uncertain and controversial. Inhalation of high concentrations of PDE is also irritating to the lung. Lifetime inhalation exposures of rats to high concentrations of PDE causes chronic lung disease and an increase in lung tumors associated with the soot. Identical exposures are not carcinogenic in Syrian hamsters, and produce equivocal results in mice. Similar tumor responses of rats to PDE soot and mutagen-poor carbon black suggest that the organic mutagens are not important in the rat response. The responses of rat and monkey lungs to inhaled PDE soot differ, and it is now considered unlikely that the rat lung tumor results are applicable to humans. There are no epidemiological studies of inhaled BDE, and no published inhalation carcinogenicity studies. At this time, the relative health effects of Biodiesel and petroleum diesel exhaust can best be estimated by comparing the soot mass produced, the mutagenicity of organic emissions, and the irritant potentials of the two materials when inhaled. BDE contains less soot mass than PDE, and a greater portion of BDE soot is soluble. The soluble organic fraction of BDE soot is less mutagenic than that of PDE soot. These differences suggest that the carcinogenic potential of inhaled Biodiesel exhaust is probably less than that of petroleum diesel exhaust. Little is known about the relative irritant potentials of inhaled Biodiesel and petroleum diesel exhaust. Depending on the fuel, engine, and operating conditions, irritating gases and vapors can be greater or lesser for BDE than for PDE. The persistence and irritancy of BDE soot in the lung should be tested in animals. Overall, our present information suggests that the health risks of Biodiesel might be less than for petroleum diesel, and that the future of Biodiesel fuels will be limited more by economic factors than by health concerns.

Introduction

Engines burning petroleum diesel fuels have been in general use for many decades, and questions continue to be raised by the public and regulatory authorities concerning the potential adverse health effects of occupational and environmental exposures to inhaled petroleum diesel exhaust. If Biodiesel fuels, diesel fuels derived from plant and animal oils, are to be successfully introduced into more general use, questions concerning the potential health impacts of inhaling Biodiesel exhaust and the comparative toxicity of Biodiesel and petroleum diesel exhausts must be addressed. The purpose of this paper is to present a summary review of the potential health effects of Biodiesel exhaust. There have been few studies of the toxicity of Biodiesel exhaust, but several studies of the composition of Biodiesel emissions. Therefore, this review presents a preliminary assessment of Biodiesel health issues based primarily on the comparative compositions of Biodiesel and petroleum diesel emissions.

Health effects of inhaling petroleum diesel exhaust

Our current understanding of the nature and potential health risks of inhaled petroleum diesel exhaust (PDE) was recently summarized well by the Health Effects Institute [1]. Concerns for the health effects of PDE have largely been focused on the risk for lung cancer from inhaling soot. Petroleum diesel soot consists of a carbon core to which a complex mixture of organic compounds, sulfates, nitrates, and metals is adsorbed. Soot particles in diluted exhaust are almost all less than one μm in diameter and are thus readily inhaled [2]. Approximately 20 to 30 % of the particles in the size range of PDE soot would be expected to deposit in the lung and airways when inhaled by humans [3]. The soluble (solvent-extractable) organic fraction (SOF) consists of aliphatic, heterocyclic, and polycyclic aromatic hydrocarbons (PAHs), and their derivatives, among which are several known mutagens and carcinogens. Concern for potential chemical-induced cancer risk from inhaled diesel soot arose in the mid 1950s when Kotin *et al.* [4] found that the SOF caused cancer when applied to mouse skin. This concern was elevated in the late 1970s when U.S. Environmental Protection Agency (USEPA) researchers reported that the SOF was a direct-acting mutagen in the Ames *Salmonella* assay [5]. These findings, and a predicted increase in diesel engine use in light-duty vehicles, stimulated a world-wide research effort to define lung cancer risk which continues today.

Epidemiology

Between 1957 and 1993, 34 epidemiological studies either focused on lung cancer risk from inhaled PDE or contained information from which some measure of risk from PDE could be inferred [1]. This body of information includes 18 cohort studies and 16 case-control studies. To a degree which varied from study to

study, these investigations suffered from two important limitations. First, none of the studies included actual measurements of the subjects' exposure to PDE soot. Lung cancer risks were estimated using surrogate estimates of exposure which included:

1. measures of particle concentrations in contemporary railroad environments;
2. the amount of diesel fuel used on shipping docks;
3. qualitative information from the subject or family about exposure to PDE;
4. records of job history; or
5. union membership.

None of these methods can be expected to provide highly accurate estimates of exposure. The second limitation is the control of the lung cancer results for smoking behavior. Cigarette smoking is known to be a very strong risk factor for lung cancer, and lack of control or inadequate control for smoking could result in smoking-related cancers being wrongly ascribed to PDE exposure. Only approximately one-half of the studies attempted to control for cigarette smoking at all, and the degree to which the controls in other studies were successful in eliminating smoking as a confounding variable continues to be debated.

The results of the epidemiological studies have been summarized thoroughly in previous publications [1, 6]. In aggregate, the results suggest that prolonged occupational exposure to high concentrations of PDE may cause a small increase in lung cancer risk, although not all studies were positive. The most robust studies published through 1993 indicated relative risks for lung cancer in the range of 1.2 to 1.6 for railroad workers (figure 1) and truck drivers.

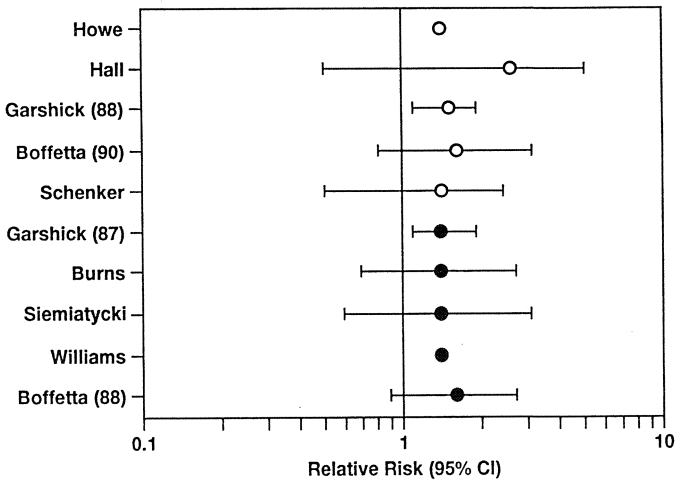

Figure 1. Relative risks for lung cancer reported from 10 cohort and case-control epidemiological studies of railroad workers. The bars indicate the 95 % confidence intervals of the estimated relative risks. Reproduced with permission from the Health Effects Institute [1].

Although the best statistical estimates of relative lung cancer risks were generally greater than 1.0 in these populations, two points should be recognized in addition to the difficulties caused by the uncertainty of the exposure estimates and controls for smoking. First, the magnitudes of the suggested increases in risk were small, ranging from approximately 20 % to 60 % above the normal, or control, risk. For comparison, it can be noted that long-term cigarette smoking increases lung cancer risk approximately 1000 % above normal, or a relative risk of 10. Even a two-fold increase in risk, or a relative risk of 2.0, would be near the lower margin of health impact that epidemiological studies can be expected to identify reliably. Second, the lower bounds of the 95 % confidence intervals shown in figure 1 often included the value of 1.0, or no increase in cancer risk. Epidemiology studies are generally not considered to show a significant increase in risk if the lower bound of the 95 % confidence interval includes the value of 1.0. For epidemiology to resolve conclusively whether or not the 20-60 % increases in risk, or relative risks of 1.2-1.6, observed in the studies of PDE are significant, new studies will probably need to be conducted with large numbers of subjects and better measures of exposure and more certain controls for smoking than studies published previously.

Laboratory Research

Laboratory research on the carcinogenicity of PDE has focused on determining:
1. the compounds responsible for the mutagenicity of the SOF;
2. the release of these compounds from soot after inhalation (bioavailability); and
3. the pulmonary carcinogenicity in animals exposed chronically by inhalation.

Fractionation of the SOF and testing in *in vitro* bacterial and mammalian cell assays has shown that the majority of mutagenic activity is contributed by a minority of the SOF mass, principally by the PAHs. Although several mutagenic compounds have been identified, the nitro-PAHs appear to be the most potent class of SOF mutagens *in vitro* [7] and have also been shown to cause mutations *in vivo* [1]. The bioavailability of SOF mutagens from soot deposited in the lung remains in question. In general, the SOF is poorly extracted by biological fluids [6, 8]. However, some mutagenic activity can be extracted from soot using lung fluid simulants [9] and mutagenic activity is released from pulmonary macrophages after ingestion of particles coated with organic compounds [10]. Experiments have shown that adsorption of organic compounds to carbon particles prolongs their release in the lung and thus sustains the dosing of cells longer than if the compounds were not attached to particles [11]. Our present understanding of the bioavailability of the SOF is not adequate to develop accurate estimates of the cellular dose of mutagens.

Numerous studies of rodents exposed chronically by inhalation to PDE have been conducted in Europe, the U.S., and Japan. Between the late 1970s and mid-1980s, 11 studies of rats were conducted in 9 laboratories, 5 studies of Syrian hamsters were conducted in 4 laboratories, and 5 studies of mice were conducted in 5 laboratories [6]. These inhalation bioassays demonstrated that DE, inhaled repeatedly at high concentrations for 24 months or longer, causes increased

incidences of lung tumors in rats. Exposures sufficient to cause lung tumors deposited soot in the lung at rates exceeding the capacity of macrophage-based particle clearance, resulting in a progressively increasing lung burden of soot. These exposures also caused chronic inflammation, focal fibrosis, proliferation of alveolar epithelial cells, and metaplasia of epithelial cells. Exposures of rats low enough to avoid substantial build-up of soot in the lung did not increase the incidence of lung tumors or cause the other types of pathology. Studies of rats exposed to exhaust filtered to remove soot demonstrated that the soot was required for carcinogenesis.

Similar exposures of Syrian hamsters did not cause an increase in lung tumors. Soot accumulated in hamster lungs and caused lung pathology, but hamsters did not develop lung tumors. The results from mice were mixed. Increased incidences of lung tumors were produced in female Sencar or Strain A mice, strains that are particularly sensitive to chemical carcinogenesis, but results were equivocal or negative in other strains. There have been no studies in which non-rodent animals have been exposed to high concentrations of PDE for a major portion of their life span; thus, the rat is the only species in which PDE has been demonstrated reproducibly to be a pulmonary carcinogen.

By the mid 1980s, a growing body of information suggested that the rat's lung tumor response to PDE soot might be a nonspecific effect unrelated to chemical carcinogenesis from the SOF. Rats were found to develop lung tumors when exposed heavily by inhalation or intratracheal instillation to many chemically and physically diverse, poorly soluble, nonfibrous particles, including several with no organic mutagens [12]. Like PDE soot, all of these particles caused lung inflammation, fibrosis, alveolar epithelial proliferation, and epithelial metaplasia in rats exposed sufficiently to overwhelm normal particle clearance and cause lung tumors. The term „lung overload" became commonly used to describe this phenomenon. These findings suggested that PDE-induced rat lung tumors might have little to do with the organic fraction of soot, and might not be predictive for cancer risk in humans inhaling PDE at much lower concentrations and depositing much less soot per unit of lung tissue.

During the early 1990s, two laboratories, the Lovelace Inhalation Toxicology Research Institute in the U.S. and the Fraunhofer Institute for Toxicology and Aerosol Research in Germany, compared the carcinogenicities of PDE soot and carbon black to examine the importance of the SOF in the tumor response of rats to PDE [13, 14]. The carbon black was used to simulate PDE soot with very little SOF and insignificant chemical mutagenic activity. Although designed differently, these studies both demonstrated that PDE soot and carbon black had similar carcinogenic potential in rats exposed heavily by inhalation, confirming that the SOF of PDE is apparently not important to the rat response. Continued research has shown that the rat is much more sensitive than other rodents to pulmonary carcinogenesis from inhaled particles [15, 16]. This information and the growing knowledge that the lungs of rats appear to respond differently than lungs of primates to inhaled particles [17], suggests that the carcinogenicity of inhaled PDE in rats should probably not be used to predict human lung cancer risk, and especially risk from much lower exposures.

Non-cancer effects

Lung cancer is not the only concern for inhaled PDE. Studies of workers have shown small decrements of lung function during workshifts involving substantial exposure to PDE, and there is some indication that long-term occupational exposure to PDE may increase the incidence of mortality and morbidity from non-cancer respiratory disease [1, 6, 18]. Short-term experimental exposures of normal humans to high concentrations of PDE have caused slight lung inflammation, as indicated by increases in inflammatory cells in airway fluid [19]. Short-term exposures of rodents to PDE have shown that inflammation can be detected within hours and increased cell division can be observed within a few days of initiating exposure [20]. Observations during the long-term cancer studies of rodents have shown that inhalation exposure to high concentrations of PDE causes lung inflammation, fibrosis, and cell proliferation, and that there is probably an exposure concentration threshold for the chronic effects [21, 22]. Comparisons of rats and mice during chronic exposure demonstrated that rats had stronger inflammatory, proliferative, and fibrotic responses [21]. Rats have also been observed to have stronger noncancer pulmonary responses than mice to other inhaled particles [22]. There are few studies of the inflammatory effects of PDE in non-rodent species. A recent comparison of the long-term accumulation of PDE soot and coal dust in lungs of rats and monkeys, and their corresponding tissue responses, demonstrated that particles accumulate differently and are predominantly retained in different anatomic locations in rats and monkeys, and that the rats have a much greater tendency toward epithelial proliferation [17].

Health effects of inhaling Biodiesel exhaust

There is much less information on the potential health effects of Biodiesel exhaust (BDE) than for PDE. There has not been sufficient use of diesel fuels derived wholly or in part from plant or animal oils to allow epidemiological studies of health effects in humans, and there are no published studies of animals exposed by inhalation to BDE. There is a growing body of information on the characteristics of emissions from engines burning Biodiesel fuels, mostly derived from rapeseed and soybean oils, and there are some data on the bacterial mutagenicity of the SOF of Biodiesel soot. Although there are still great uncertainties about the human health effects of PDE, the concerns for PDE continue to focus on the characteristics of soot emissions, including soot mass and mutagenic activity, and on the irritant, or inflammatory, potential of inhaled emissions. At this time therefore, one can probably best estimate the relative health risks of BDE and PDE by comparing the physical, chemical, mutagenic, and irritant natures of the two materials. An exhaustive review of BDE will not be attempted in this paper; the accompanying papers in this volume provide more detailed summaries of current knowledge. The examples given below are generally characteristic of BDE from burning plant oil-derived fuels, and allow

preliminary speculation about the comparative potential health risks of BDE and PDE.

Mass emissions and characteristics of Biodiesel soot

In most comparisons using identical diesel engines and operating conditions, Biodiesel fuels and blends have produced lower concentrations of soot in exhaust than produced by petroleum diesel fuel. Mittlebach *et al.* [23] reported emissions from a 2.3L turbocharged, four-cylinder, four-stroke, direct injection engine with exhaust gas recirculation operated in a 1360 kg vehicle on a chassis dynamometer on the U.S. Federal Test Procedure (US-75) urban cycle. Five fuels were compared:

1. U.S. D-2 petroleum diesel with high aromatic (40 %) and sulfur (0.4 %) contents;
2. U.S. D-2 petroleum diesel with lower aromatic (30 %) and sulfur (0.26 %) contents;
3. currently available Austrian petroleum diesel (32 % aromatics, 0.52 % sulfur);
4. raw rapeseed oil methyl ester (RME); and
5. refined RME.

As shown in figure 2, the total soot mass emissions for the unrefined RME were equivalent to those from the low aromatic/sulfur D-2, and less than those from the other petroleum diesel fuels. Soot mass emissions for the refined RME were higher than those from the low aromatic/sulfur D-2, similar to those from the Austrian petroleum fuel, and lower than those from the high aromatic/sulfur D-2.

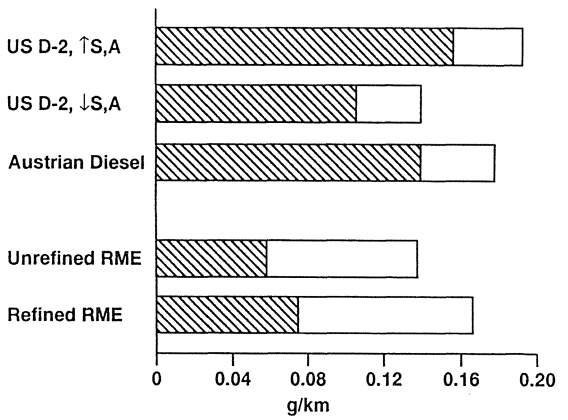

Figure 2. Soot emissions from engine burning petroleum diesel fuel with high sulfur and aromatic content (US D-2, ↑ S,A), petroleum diesel with lower sulfur and aromatic content (US D-2, ↓ S,A), a commercial Austrian fuel, or unrefined or refined rapeseed oil methyl ester (RME). The hatched portion indicates the insoluble, carbonaceous portion of soot, and the clear portion indicates the soluble organic fraction (SOF). From Mittlebach *et al.* [23].

McDonald *et al.* [24] reported emissions from a 7.0L Caterpillar Model 3304, naturally aspirated, indirect injection mine engine operated on the ISO 8178-C1 eight-mode off-highway cycle. Three fuels were used:

1. a U.S. D-2 petroleum diesel fuel with 30 % aromatics and 0.04 % sulfur;
2. soybean oil methyl ester (SME); and
3. a 70 % D-2, 30 % SME blend.

With each fuel, emissions were measured with and without treatment with an oxidation catalyst. As shown in figure 3, the SME blend and 100 % SME fuels produced progressively less soot mass than the D-2 fuel, both with and without the catalyst.

Shumacher *et al.* [25] operated a Model 6V92TA 9.03L Detroit Diesel Corporation direct injection engine with a catalytic converter on the USEPA heavy duty engine test cycle burning either No. 2 low sulfur petroleum diesel fuel or this fuel blended with 10 %-40 % soybean-derived Biodiesel fuel. These investigators reported that blending with Biodiesel reduced soot and hydrocarbon emissions. They also found that the tendency for increased nitrogen oxides with Biodiesel blends could be controlled by retarding fuel injection timing, and that 20 % Biodiesel was the optimum blend for minimizing emissions.

It is important to recognize that, although Biodiesel fuels have generally been reported to produce less soot mass than petroleum diesel, this is not always the case. The extensive testing program reported by Pischinger and Sams [26] included evaluation of emissions from a 2.47L Deutz engine and a 9.7L Steyer engine on test stands, and a 6.87L MAN engine operated in a bus on a chassis dynamometer. The engines burned either petroleum diesel or RME, and exhaust was measured with and without treatment by oxidation catalyst. The comparative emissions between RME and petroleum diesel varied, depending on the engine and operating condition. Under some conditions, soot mass emissions were higher with RME fuel. Similarly, Kado *et al.* [27] found that either petroleum diesel or rapeseed oil ethyl ester produced higher mass emissions of soot when burned in a Cummins 5.9L turbo diesel, depending on operating conditions. The Biodiesel fuel produced less soot under cold start conditions, but more under hot start conditions. Krahl *et al.* [28] reviewed the nature and potential environmental effects of emissions from engines burning petroleum diesel fuel, rapeseed oil, and RME. They reported that in an indirect injection engine operated on an urban cycle, emissions of soot and hydrocarbons were higher for rapeseed oil than for petroleum diesel, but nitrogen oxides were similar. Emissions of soot were lower and nitrogen oxides were higher for RME than for petroleum diesel, but hydrocarbons were similar.

The type of engine is an important consideration, as well as the fuel and operating conditions. No engines have yet been developed to optimize the work produced and minimize the emissions from Biodiesel fuels; thus, emissions reported in the literature are often produced by engines that are not as well-suited for Biodiesel as for petroleum diesel fuel. Based on current literature, it is not possible to make a conclusive statement about the relative soot mass emissions from petroleum diesel and Biodiesel fuels because either fuel could produce the higher emissions under selected conditions of use.

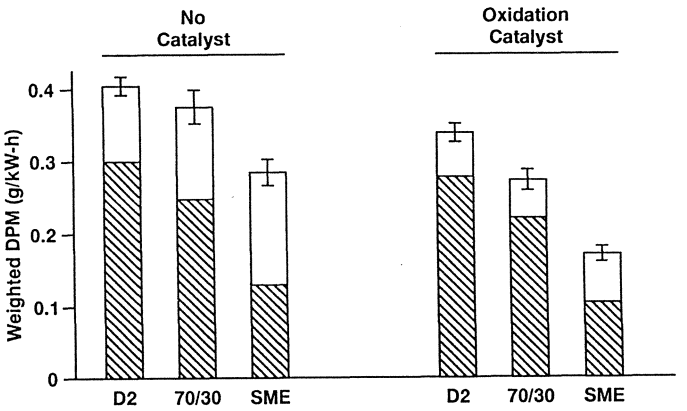

Figure 3. Soot emissions from engine burning petroleum diesel fuel (D2), soybean oil methyl ester (SME) or a blend of 70 % D2 and 30 % SME, either with or without the use of an oxidation catalyst on the exhaust system. DPM = diesel particulate matter. The hatched portion of the bars indicate the non-volatile portion of soot, and the clear portion indicates the volatile portion. From McDonald *et al.* [24].

It seems to be a consistent finding, however, that soot from the combustion of plant oil-derived Biodiesel fuel always contains a greater percentage of SOF and a lesser percentage of insoluble mass than petroleum diesel soot. This relationship can be seen in the results of Mittlebach *et al.* [23] in figure 2 and in the results of McDonald *et al.* [24] in figure 3, and was also observed in all comparisons reported by Pischinger and Sams [26]. The health implications of the higher SOF of Biodiesel soot are not known at this time. Information from petroleum diesel soot is of little help, because there is not sufficient information to judge whether or not differences in the portion of SOF affect the toxicity of petroleum diesel soot. It is thought that the persistence and accumulation of insoluble material contributes strongly to the toxicity of particles in the rat lung [15]. In the rat lung, therefore, the toxicity of Biodiesel soot might be less because a greater portion of the soot is SOF which should dissolve more readily in the lung. It is not clear what role the persistence of solid particles, and thus particle solubility, might play in soot toxicity in other species. On the other hand, if the mutagenicity or other toxicity of Biodiesel soot SOF is equal to, or greater than, that of petroleum diesel soot SOF, the greater portion of Biodiesel soot SOF might give it a greater toxicity. The persistence and toxicity of Biodiesel soot in the lung is not known at this time and should be explored.

Mutagenicity of the SOF of Biodiesel SOF

There are several reports of the mutagenic potential of Biodiesel soot SOF, but there is not yet sufficient information for a good understanding of the influences of fuel and engine type and operating condition on mutagenicity. Results

published at this time suggest, however, that Biodiesel SOF generally has a lower mutagenic activity per unit of mass (specific mutagenicity) than petroleum diesel SOF. This is illustrated by the results of Stalder *et al.* [29], which are summarized in figure 4. Stalder *et al.* used the Ames bacterial mutagenicity assay to test the SOF of soot collected from a Fendt Farmer direct injection tractor engine burning either petroleum diesel fuel or RME, and found that the Biodiesel SOF had much lower specific mutagenicity in two tester strains of bacteria. An example of the integrated effect of the greater SOF content and lower SOF mutagenicity of Biodiesel compared to petroleum diesel can be observed in the results of Kado *et al.* [27]. These authors reported that, although the soot mass was greater for RME than for petroleum diesel under some conditions, the total mutagenic activity produced per mile of simulated vehicle use was always lower for RME than for petroleum diesel fuel.

Irritant potential of Biodiesel emissions

There are no published studies of the irritant or inflammatory effects of BDE inhaled by animals or humans; thus, the irritant potential of BDE can only be speculated. Certainly, BDE can be supposed to have some irritating properties because it contains irritant gases and vapors, such as nitrogen oxides and aldehydes, and a wide range of organic compounds, some of which are likely to be irritating. At this time, we can best speculate about the irritant potential of BDE by comparing its composition to PDE. Such a comparison results in a mixed view.

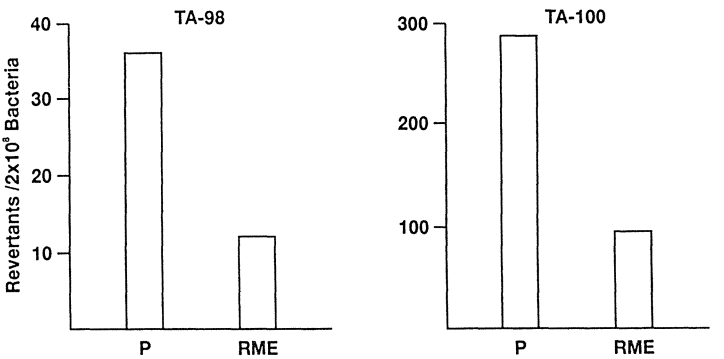

Figure 4. Mutagenicity of the solvent extract of soot from an engine burning either petroleum diesel fuel (P) or rapeseed oil methyl ester (RME). Bacterial mutagenicity of the extract was tested in the Ames assay using two tester strains of *Salmonella*, TA-98 and TA-100. The mutagenicity indicated in this figure was direct-acting, because the assay was conducted without the addition of microsomal enzymes for metabolism of the organic compounds. From Stalder *et al.* [29].

Oxides of nitrogen emissions are sometimes higher for BDE than for PDE [23, 26, 28], but not always [24, 28]. Concentrations of aldehydes and acrolein [23] and of certain organic compounds [26, 27] are sometimes higher in BDE than in PDE. These relationships depend on the engine and operating conditions. Engines optimized for burning Biodiesel fuel have lower emissions than when optimized for petroleum diesel fuel. Overall, the irritant potential of BDE is uncertain and should be evaluated more directly by inhalation studies.

Summary

The small amount of information on the health effects of BDE at this time, and the diverse and changing combinations of fuels and engines evaluated to date do not allow definitive conclusions about the potential of BDE for inducing adverse health effects. Based on the above comparisons to PDE however, it is possible to make some general preliminary statements about the probable relative effects of BDE and PDE. The emissions of soot mass and mutagenic activity are generally less for engines burning Biodiesel fuel than for petroleum diesel fuel. These factors suggest that BDE is likely to cause no more health risk, and may cause less health risk, than PDE. The irritant potential of BDE has not been directly explored; thus, little can be speculated about the relative potential of BDE for irritant and inflammatory effects. Both the persistence of BDE soot in the lung and the irritancy of BDE could be explored in straightforward inhalation studies in animals, and such studies are recommended.

Overall, our present information suggests that the development and use of Biodiesel fuels are not likely to be limited by health concerns. This should not be taken to suggest that there would be no adverse health impact of breathing high concentrations of Biodiesel exhaust. Rather, it suggests that Biodiesel exhaust is not likely to be of greater concern, and will probably be of less concern, from a health viewpoint than petroleum diesel, the fuel Biodiesel would principally displace. At present, it appears that the use of Biodiesel fuels will be driven more by economics than by health concerns.

References

1. HEI, "Diesel exhaust: A critical analysis of emissions, exposure, and health effects," A Special Report of the Institute's Diesel Working Group (Health Effects Institute, Cambridge, MA, 1995).
2. Y. S. Cheng, H. C. Yeh, J. L. Mauderly, B. V. Mokler, *Am. Ind. Hyg. Assoc. J.* **45**, 547 (1984).
3. R.B. Schlesinger, in *Concepts in Inhalation Toxicology*, R. O. McClellan and R. F. Henderson, Eds. (Taylor & Francis, Washington, DC, ed. 2, 1995), chap. 8, pp. 191-224.
4. P. Kotin, H. L. Falk, M. Thomas, *Ind. Health* **11**, 113 (1995).

5. J. L. Huisingh *et al.*, in *Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures,* M. D. Waters *et al.*, Eds. (Plenum Press, New York, NY, 1978), pp. 381-418.
6. J. L. Mauderly, in *Environmental Toxicants: Human Exposures and Their Health Effects*, M. Lippmann, Ed. (Van Nostrand Reinhold, New York, NY, 1992), pp. 119-162.
7. International Agency for Research on Cancer, "Diesel and gasoline engine exhausts and some nitroarenes" (World Health Organization, Lyon, France, 1989), pp. 189-373.
8. A. L. Brooks *et al.*, *Environ. Intl.* 5, 263 (1981).
9. M. J. Keane, S-G. Xing, J. C. Harrison, T. Ong, W. E. Wallace, *Mutat. Res.* 260, 233 (1991).
10. J. A. Bond, M. M. Butler, M. A. Medinsky, B. A. Muggenburg, R. O. McClellan, *J. Toxicol. Environ. Health* **14**, 181 (1984).
11. J. D. Sun, R. K. Wolff, S. M. Maio, E. B. Barr, *Inhal. Toxicol.* **1**, 1 (1989).
12. J. L. Mauderly, Y. S. Cheng, M. B. Snipes, *J. Aerosol Med.* **3(Suppl 1)**, 169 (1990).
13. U. Heinrich *et al.*, *Inhal. Toxicol.* **7**, 553 (1995).
14. K. J. Nikula *et al.*, *Fundam. Appl. Toxicol.* **25**, 80 (1995).
15. J. L. Mauderly and R. J. McCunney, Eds., *Particle Overload in the Rat Lung and Lung Cancer: Implications for Human Risk Assessment* (Taylor & Francis, Washington, DC, 1996).
16. J. L. Mauderly, *Environ. Health Perspect.,* **105**, 1337 (1997).
17. K. J. Nikula, W. C. Griffith, K. J. Avila, J. L. Mauderly, *Fundam. Appl. Toxicol.*, **37**, 37 (1997).
18. J. L. Mauderly, *Environ. Health Perspect.* **102**, 165 (1994).
19. B. Rudell, T. Sandström, N. Stjernberg, B. Kolmodin-Hedman, *J. Aerosol Sci.* 21, S411 (1990).
20. H. J. White and B. D. Garg, *J. Appl. Toxicol.* **1**, 104 (1981).
21. R. F. Henderson *et al.*, *Fundam. Appl. Toxicol.* **11**, 546 (1988).
22. J. L. Mauderly, in *Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract* (ILSI Press, Washington, DC, 1994), pp. 43-55.
23. M. Mittelbach, P. Tritthart, H. Junek, *Energy Agric.* **4**, 207 (1985).
24. J. McDonald, D. L. Purcell, B. T. McClure, D. B. Kittelson, *SAE Report 950400* (Presented at the International Congress, Detroit, MI, February 27, 1995), pp. 1-17.
25. L. G. Schumacher, S. C. Borgelt, D. Fosseen, W. Goetz, W. G. Hires, *Bioresource Technol.* **57**, 31 (1996).
26. R. Pischinger and Th. Sams, "Emissionsverhalten bei realem RME-Betrieb mit bzw. ohne Oxikat" (Bundesministerium für Land- und Forstwirtschaft und des Bundesministerium für Wissenschaft, Forschung und Kunst, Austria, Vienna, 1995).
27. N. Y. Kado, R. A. Okamoto, P. A. Kuzmicy, "Chemical and bioassay analyses of diesel and biodiesel particulate matter: Pilot study," Final Report (Department of Environmental Toxicology, University of California Davis, CA, 1996).
28. J. Krahl, A. Munack, M. Bahadir, L. Schumacher, N. Elser, in *Topics in Alternative Fuels and Their Emissions* (Society of Automotive Engineers), 1208, 311 (1996).
29. K. Stalder, V. Gerhard, J. Krahl, A. Munack, *Landtechnik* **50**, 266 (1995).

Biodiesel Exhaust Emissions and Determination of their Environmental and Health Effects

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Abstract

In Europe, the use of rapeseed oil fuels in diesel engines has been intensively investigated since the energy crisis of the early 1970s. In the beginning, the emphasis was placed on the technical possibilities associated with the use of rapeseed oil as a fuel. However, research has shown that pure rapeseed oil can only be used in specially designed engines. Research that followed indicated that rapeseed oil methylester (RME) was a suitable replacement for petroleum diesel fuel (DF) [30]. After this discovery, research has focused on the engine exhaust emissions that result when fueling with both unmodified rapeseed oil and RME [14]. In the USA research has focused on soybean oil methylester (SME). Both RME and SME are called Biodiesel. In Germany Biodiesel must fulfill the standard DIN V 51606.

Initially, environmental related research concentrated on the federally regulated hydrocarbons (HC), carbon monoxide (CO), and oxides of nitrogen (NO_x) exhaust gas emissions [31]. In addition, a series of current publications compare the environmentally important but non-regulated polycyclic aromatic hydrocarbons (PAH), aldehydes, ketones and in some cases, the aromatic compounds.

Introduction

An estimation of the environmental effects caused by emissions from engines fueled with rapeseed oil methylester can be made by a relative comparison with DF. An evaluation of the potential health effects, as presented for gasoline and diesel-powered engines in an environmental assessment of the „Sachverständigenrat für Umweltfragen" (German Authority Council for Environmental Issues) [23], must be replicated for RME in the future.

The goal of this review is to summarize the published emissions measurements from different authors, to compare these results and, where possible, identify

trends that may exist. In order to classify and evaluate the conclusions, it is necessary to begin with a description of the experimental conditions applied, or in other words, the engine testing procedures.

Engine testing procedures

The engine test procedures must be selected carefully. Engines emit different levels of harmful substances depending upon load and engine speed. A comparative evaluation of the engine exhaust emissions is only possible when specific engine testing procedures are employed. For automobiles, test cycles that contain city and highway test conditions should be used. The test cycles that are used most frequently include the American Federal Test Procedure (FTP) and the European ECE-13 Test (ECE = Economic Commission for Europe). The ECE test procedure now includes a section that measures emissions at higher speeds to adjust to the traffic conditions that exist in Europe and is denoted MVEG-A Test (Motor Vehicles Emissions Group). The results of exhaust gas emissions from these test procedures are not directly comparable, as the driving cycles load the engines differently [24].

Worldwide many test procedures are available for trucks. In the EU (EU = European Union), truck emissions are analyzed using the 13-mode steady-state test cycle ECE R49. In contrast to the U.S. transient cycle, this steady state truck test takes into consideration the European driving style and the European engine design of the vehicles [5]. Diagram 1 illustrates the selected operating points used during the engine emissions test procedure and the weighting factors for each part of the test.

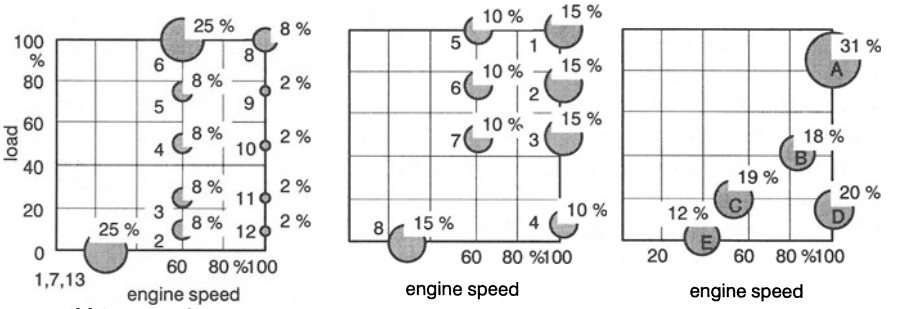

Diagram 1. The load points of the 13-, 8- and 5-mode tests

Truck and car test cycles are not suitable to judge the engine exhaust from agricultural tractors. The simulated city and highway tests are not appropriate for agricultural vehicles, because the engines are loaded differently. The steady-state 5-mode test developed by Vellguth [31] has been adjusted for emissions testing of agricultural engines. This emissions test procedure is based on research conducted by Welschof [32], whereby 5 modes accurately represent the typical load and speed profile of agricultural vehicles.

The International Standards Organization (ISO) defined an 8-mode test to certify agricultural engines. This test is based on the European 13-mode test. As is evident in diagram 1, only two engine speeds in addition to idling are considered. It is uncertain that if the cycle recommended in the ISO 8178-C1 can be relied upon not only for certification but also to accurately estimate the exhaust emissions actually produced by agricultural use.

In conclusion, in view of the comparability of the emissions values determined among the various test procedures, a direct comparison of the absolute values is not admissible. For this reason, the following results will be represented based on the engine test procedure used.

Comparison of emissions from rapeseed oil, RME and Diesel fuel

Gaseous regulated exhaust gas components, particulate matter, and soot number

Many publications are available that describe comparative measurements of 100 % DF and RME. Because the quantifications of HC, CO, and NO_x are usually undertaken with reliable and commercially available gas analyzers, the discussion of the analytical procedures and their results that are used to determine relative changes in these components is unnecessary. Likewise, standardized methods with good reproducibility exist for determination of particulate matter and soot number. In constructing the following summary, we have referenced current secondary literature [12,25] related to the topic. However, the quoted relative changes in emissions must be corrected in some cases after the original report was reviewed as the data were inaccurately repeated.

Diagrams 2 to 7 show the relative differences in values for regulated emissions compounds and soot number compared to DF. The distinction is made here according to the engine test procedure and engine design (IDI = engine with indirect injection; DI = engine with direct injection).

In summary, the comparison of steady state and transient test cycles shows no significant variations. The use of DI or IDI engines did not reveal any significant advantages for either engine type.

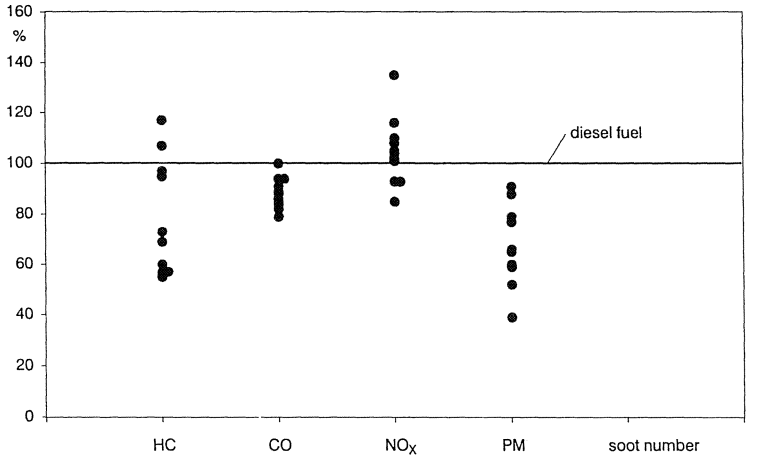

Diagram 2. Emissions of regulated compounds in IDI engines fueled with RME compared to petroleum diesel fuel in the FTP-75 test (DF = 100 %)

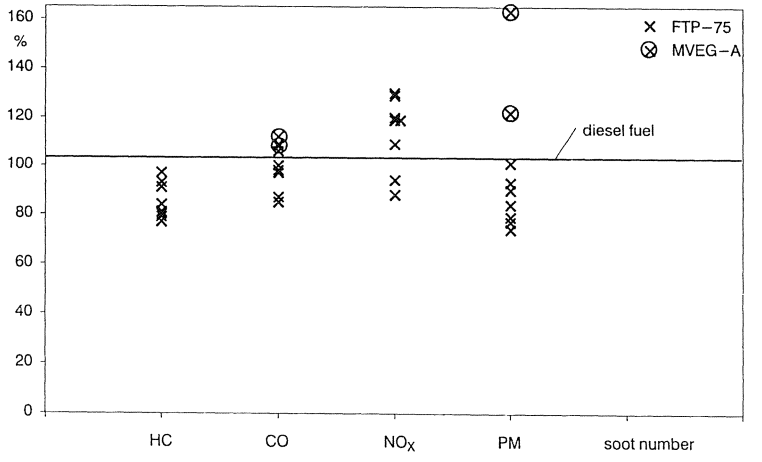

Diagram 3. Emissions of regulated compounds in DI engines fueled with RME compared to petroleum diesel fuel in the FTP-75 and the MVEG-A test (DF = 100 %)

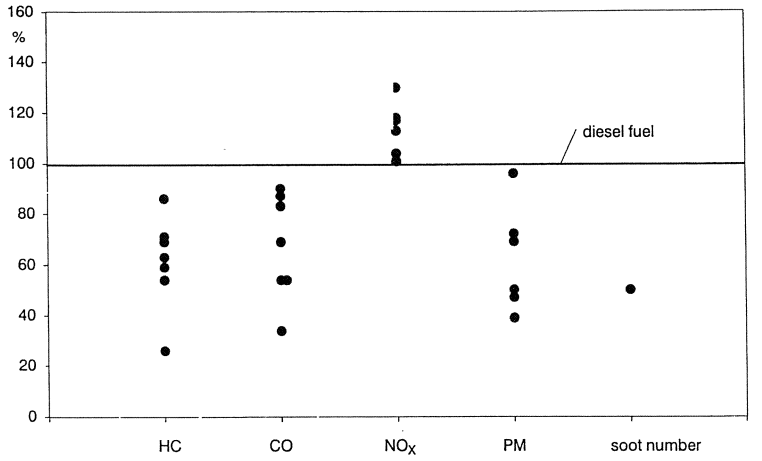

Diagram 4. Emissions of regulated compounds and soot number in IDI engines fueled with RME compared to petroleum diesel fuel in the European 13-mode test (DF = 100 %)

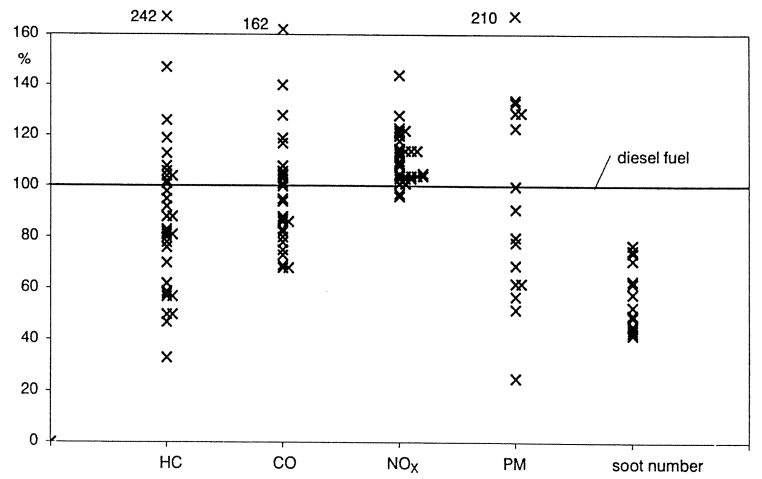

Diagram 5. Emissions of regulated compounds and soot number in DI engines fueled with RME compared to petroleum diesel fuel in the European 13-mode test (DF = 100 %)

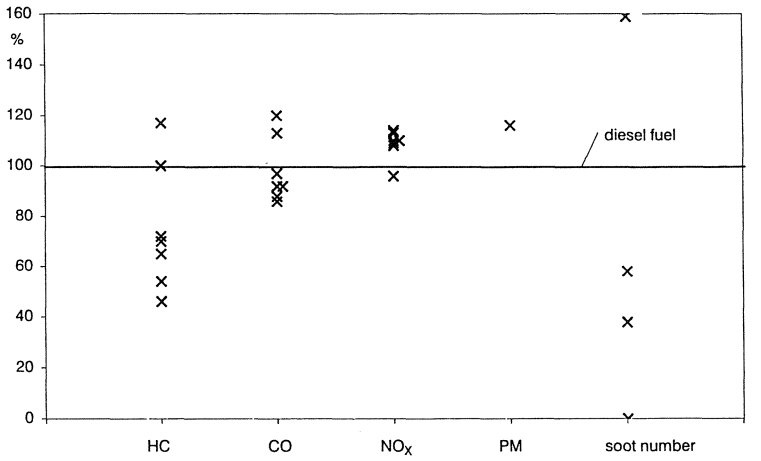

Diagram 6. Emissions of regulated compounds and soot number in DI engines fueled with RME compared to petroleum diesel fuel in the agricultural 5-mode test (DF = 100 %)

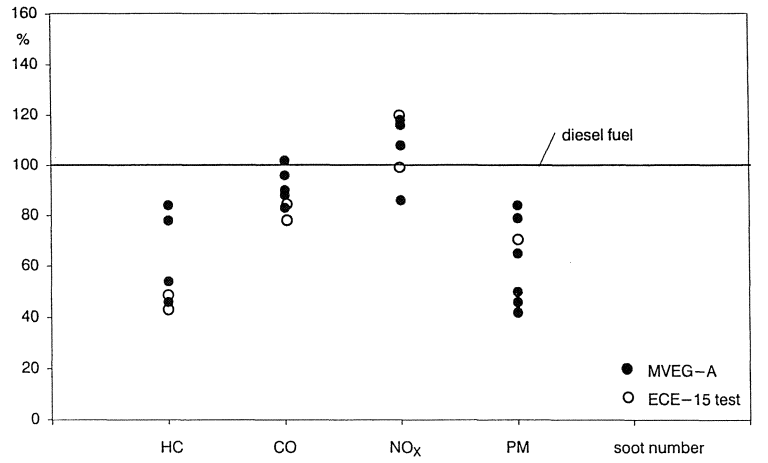

Diagram 7. Emissions of regulated compounds and soot number in IDI engines fueled with RME compared to petroleum diesel fuel using the ECE-13 and MVEG-A test procedures (DF = 100 %)

In particular, HC discharge of both engine designs was reduced by about 20 % in the FTP-75 test. For IDI engines running on RME, a HC reduction of approximately 40 % in the 13-mode test is observed, whereas this value with DI engines was decreased by only about 10 %. These observed tendencies, however, should be interpreted with caution, as a much larger database is available for DI engines.

CO was reduced on average by 13 % for all engine designs with RME use, whereby IDI engines show some advantages. NO_x emissions increased by almost 10 %. No PM changes were observed for DI engines tested with the 13-mode test with either RME or rapeseed oil. In almost all other cases, a reduction of approximately 20 to 40 % occurred when compared to DI engines when RME was used. IDI engines generally emit less particulate matter. The soot number was independent of engine design and test cycle. A reduction in soot number of approximately 40 % was noted when RME was compared to diesel fuel.

Non-regulated exhaust gas components PAH, Aldehydes, and aromatic compounds

In contrast to the numerous comparative measurements of regulated emissions, the availability of data is rather limited for the non-regulated compounds. Among the unregulated components of exhaust gas, aldehydes have been more thoroughly investigated than PAH. In isolated cases, indications of emissions of benzene and alkyl benzene derivatives have also been reported. The expenditure necessary to quantify carbonyl compounds is much lower compared to that of PAH, which explains the varying amount of information on aldehydes and PAH.

Within the framework of the following section, the analytical precision of PAH, aldehyde and aromatic compound determinations will not be discussed. Many of the PAH values are based on single measurements without repetitions. The relative deviations of the aldehyde measurements with repeated measurements range from ± 2-20 %, whereas the repeated standard deviations with multiple PAH measurements varied from 5 to 40 %. Detailed information concerning the following test results of unregulated emissions was documented by Krahl *et al.* [13], including sources, description of analytical procedures, number of repeated measurements and, when available, relative standard deviations of repeated measurements.

The following criteria were used to select data for comparison purposes: 1) common engine test cycle, 2) information can be used quantitatively according to information from the analytical laboratory. Therefore, experiments were excluded when the analytical laboratory stated a deviation of ± 100 %.

Comparative measurement of PAH

In the final result of the experiments shown in diagram 8, it is clear that fueling with RME significantly reduced PAH exhaust emissions of most engines. This decrease was especially noticeable with the 5-mode test that was designed for

agricultural engines. The investigation that led to an increase of PAH is not explainable in the moment. It has to be repeated with a similar engine under similar conditions.

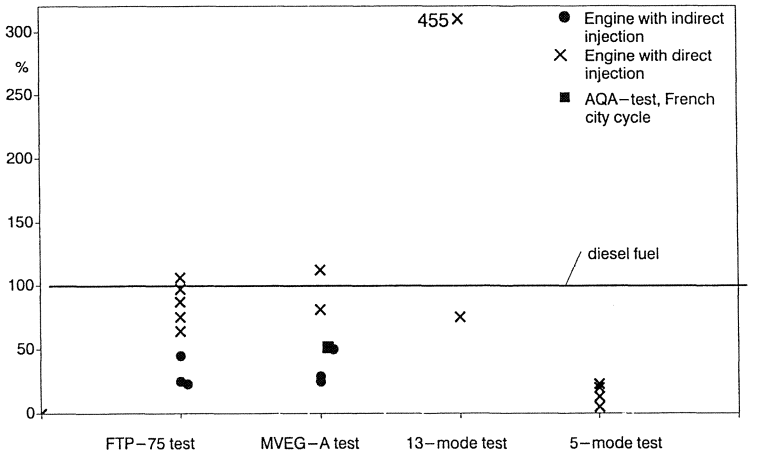

Diagram 8. Relative PAH emissions values from engines fueled with RME compared to petroleum diesel fuel (DF = 100 %)

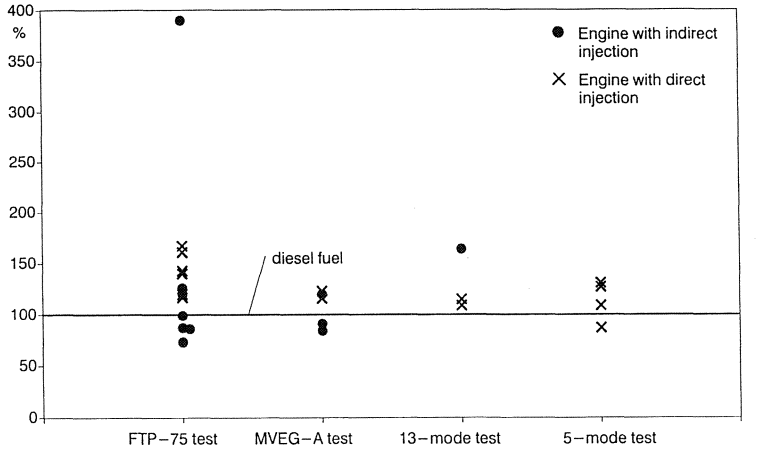

Diagram 9. Aldehydes emissions from engines fueled with RME compared to petroleum diesel fuel (DF = 100 %)

Comparative measurements of aldehydes

The relative values of aldehyde and ketone emissions are represented for the different test cycles in diagram 9. RME aldehyde emissions increased approximately 40 % compared to operation with DF. Formaldehyde and acrolein were responsible for this increase. It is interesting to note that the upper and lower extreme emissions cases were produced by the same engine manufacturer in the FTP-75 test. There is no certain explanation for this discrepancy. The emissions from one vehicle that was fueled with RME was quite high compared to the other engines that were evaluated. When this case was removed, RME produced a 20 % increase in exhaust emissions. The comparison of indirect-injected with direct-injected engines doesn't reveal any particular tendency.

Comparative measurements of aromatic compounds

The emissions of benzene, toluene, xylenes (BTX) as well as ethylbenzene have only been sparingly investigated for engines fueled with RME. Diagram 10 shows the available information in relative representation. It is clear that for the comparison of RME with DF in the FTP-75 and in 5-mode tests, a reduction of aromatic compounds emissions are found, similar to the levels noted concerning PAH emissions.

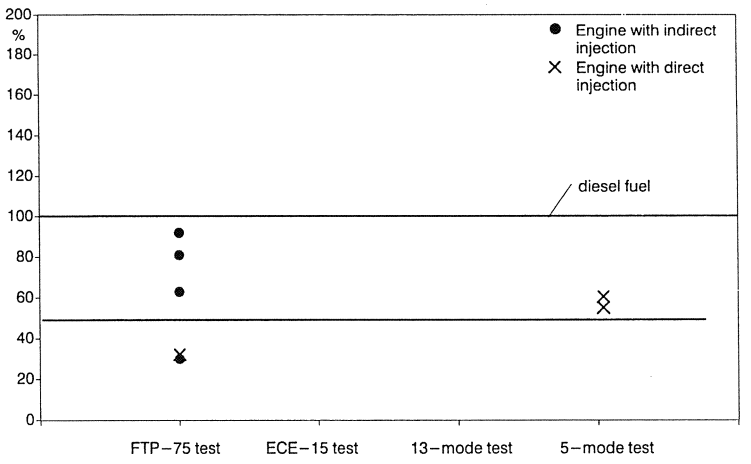

Diagram 10. Aromatic compounds emissions from engines fueled with RME compared to petroleum diesel fuel (DF = 100 %)

Total emissions of BTX and ethylbenzene correlate strongly with the measured values for benzene, an especially important compound. Diagram 11 shows the relative emission values of benzene.

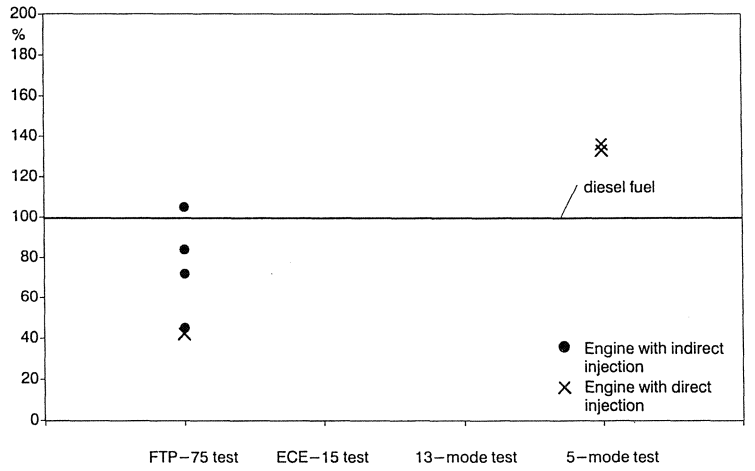

Diagram 11. Benzene emissions from engines fueled with RME compared to petroleum diesel fuel (DF = 100 %)

With the exception of the engine in the 5-mode test, similar tendencies were noted for the sum of the aromatic compounds and benzene emissions. The sharp rise in benzene emissions is significant when compared with the lower total emissions of aromatic compounds from the engine in 5-mode test. But it has to be pointed out that this discrepancy is based only on one engine. This research must be replicated to determine if this trend is real or caused by this specific probationer. However, the results obatined at FTP-75 test do not reveal such a large discrepancy.

Summary of the known test results

The influence of rapeseed oil and RME on the emission of federally regulated components has been investigated extensively. Table 1 presents a summary of average values for engines fueled with RME compared to engines powered with diesel fuel. Please note that the table does not indicate the number of measurements conducted.

Table 1: Emissions of regulated compounds and soot number in engines fueled with RME (DF = 100 %), IDI: indirect injection; DI: direct injection

Component	RME	
hydrocarbons (HC)70 %	IDI	
	80-90 % DI	
carbon monoxide (CO)	90 %	IDI
	100 %	DI
nitrogen oxides (NO_x)	110 %	
particulate matter (PM)	60-80 % IDI	
soot number	60 %	

In summarizing the following section concerning non-regulated compounds, the differentiation with respect to the engine design cannot be determined, as too little data were evaluated by the researchers. It must be noted, however, that the PAH values reported in some publications are questionable (the single reported increase to about 455 % of the diesel value was excluded for table 2).

The tendencies stated are not only based on averages of repeated measurements (range of standard deviations: 13-40 %) but also on single determinations. Table 2 provides a comparison of measurements for operation with RME relative to operation with diesel fuel. The table does not indicate the number of underlying measurements nor does it provide the accuracy of the repeated measurements; information concerning most of these factors was documented by Krahl *et al.* [13].

Table 2: Unregulated emissions from engines fueled with RME compared to petroleum diesel fuel (DF = 100 %)

Component	RME	
PAH	65 %	
	13 %	5-mode test
aldehydes	120 %	
aromates	60 %	
in particular: benzene	70 %	
	135 %	5-mode test

Estimation of environmental effects due to volatile exhaust gas components

Gaseous regulated components

No significant differences in direct effects can be deduced from changes in the gaseous regulated components. As discussed later with regard to the aldehydes an indirect photochemical effect of specific hydrocarbons may occur.

Aldehydes

The discharge of aldehydes into the atmosphere is of environmental relevance both in a direct as well as in an indirect way. In light of the direct effect of aldehydes, Wolfensberger [33], during an experiment in the city of Zurich, noted that exhaust gas fumes from buses run with RME caused some of the maintenance staff and drivers to become ill. However, the typical odor of exhaust from vegetable oil fuels was practically eliminated by an oxidative catalytic convertor (see also [15]). Stalder *et al.* [26] found in preliminary laboratory experiments that RME-soot elevated cytotoxicity. Additional systematic experiments, however, must be carried out. Aldehydes also have a great influence on tropospheric ozone

formation. Because aldehydes from diesel engine combustion eventually contribute to more than 40 % of the ozone-forming potential, the increase in aldehyde discharge deserves attention, especially when vehicles fueled by RME are used in cities. The increase of ethene and ethyne which has been determined during engine operation with RME [34] is also worthy of consideration. Together both components are suspected to contribute to over 30 % of the total formation of ozone induced by diesel engines [8].

Currently, an extended research program is carried out at the German Technical Agricultural Research Centre and at the Fraunhofer-Institute Hannover to determine both the ozone pecurser concentrations in the exhaust gases of DF and RME and their ozone forming potentials under typical German conditions by use of a smog chamber.

Aromatic compounds

Emissions of aromatic compounds tend to decrease when fueling with RME. However, they are elevated for rapeseed oil. Wurst *et al.* [34] reported a distinct increase of benzene emissions during the 5-mode test. This phenomenon cannot be satisfactorily explained yet. A need exists for research concerning the ozone-forming potential of the alkyl derivatives of benzene found in RME exhaust gas. The benzene emissions and their environmental impact should be systematically investigated at the same time.

Estimation of environmental effects due to the particulate emission

Diesel engine exhausts are classified as a carcinogen to experimental animals (sufficient evidence) and as a probable carcinogenic agent (limited evidence) to humans by the International Agency for Research on Cancer (IARC [11]). PAH and soot are considered to be responsible for this some PAH have a high carcinogenic potency syergistically with soot particles than each component alone [9]. Recently, various studies reported a relative risk of approximately 1.5 for lung cancer by diesel engine emissions (DEE) [6,7,20,22,28]. However, some authors criticized these studies for underestimating the confounding effects of cigarette smoke [21,29].

In various *in vitro* test systems DEE act as mutagens. Most investigations were done using the AMES-test [2,3,4,10]. To quantify changes of these mutagenic effects caused by the particulate of RME compared to those of DF, some *in vitro* studies were performed at the Center of Environmental and Occupational Medicine, University of Göttingen, Germany.

In vitro studies

Preliminary information on the carcinogenicity of compounds or mixtures can be obtained by determination of their mutagenic properties in *in vitro* test systems because these results very often correlate to carcinogenic effects in animals or humans [1]. Using the revised methods [18] of the *Salmonella typhimurium*/mammalian microsome mutagenicity test (AMES-test), the mutagenic effects of particle extracts of RME-emissions were directly compared to those of DF-exhausts.

Probationers and test procedures

Two probationers were examined. A passenger car Volkswagen Vento, 1.9 l TDI with oxidation catalytic converter and a tractor Fendt Farmer 306 LSA without catalytic converter. The passenger car was tested at the chassis dynamometer with exhaust gas dilution tunnel of the University of Magdeburg. For tractors a p.t.o. dynamometer is available at the Federal Agricultural Research Centre (FAL). To the time, when the investigations were performed, the FAL did not own an exhaust gas dilution system. Nowadays, this facility does.

In particular, the passenger car was tested using the U.S. FTP-75 and the European MVEG-A cycles. Additional, the emitted particulate of the cold start phase of the MVEG-A cycle was collected separately. In the following this particulate is named MVEG-A1.

The agricultural tractor was tested according to the 5-mode test

Sampling and extraction procedures

Both diluted exhaust particle samples from the passenger car and the undiluted ones from the tractor were collected under cool conditions (<50 °C) on teflon coated glass fiber filters. All filters were weighed before and after the loading and as well after extraction to determine the collected and extracted masses. The filters were stored at 5 °C and extracted afterwards with dichloromethane in a 250 ml Soxhlet apparatus for 24 h at 65 °C in the dark. The extracts were reduced by rotary evaporation and dried under a stream of nitrogen. Then, each residue was dissolved in 4 ml dimethyl sulfoxide (DMSO) for *in vitro* testing.

Mutagenicity assay

The *Salmonella typhimurium*/mammalian microsome test, published by Ames *et al.* in 1975 [1], detects mutagenic properties of a wide spectrum of chemicals and their mixtures by reverse mutations of a series of *Salmonella typhimurium* strains, bearing mutations in the histidine operon. This results in histidine requirement of the tester strains in contrast to wild-type *Salmonella typhimurium*.

This study employed the revised test protocol [18] with the recently recommended strains TA97a, TA98, TA100, and TA102. The strains TA97a [16] and TA98 detect frameshift mutagens; TA100, base-pair substitutions; TA102, oxidative mutagens [17]. Tests were performed with and without metabolic activation by a microsomal monoxygenase system (S9 fraction). Preparation of the liver S9 fraction from young male Sprague-Dawley rats was carried out as described by Maron and Ames [18]. For induction of the liver enzymes, phenobarbital and -naphthoflavone were used instead of Arochlor-1254, which is a polychlorinated biphenyl (PCB) mixture and a carcinogen of great stability [19]. The diagnostic mutagens methyl methanesulfonate (MMS) and 2-aminofluorene (2-AF) were used as positive controls.

Immediately before use, the extracts and 2-AF were dissolved in DMSO and diluted to the test concentrations.MMS was dissolved in distilled water. 2.5 ml of molten soft agar containing 0.05 mM histidine and 0.05 mM biotin were mixed with 0.1 ml of a test solution and 0.1 ml of overnight culture of the strains. After a short agitation, the mixture was poured immediately onto a minimal-agar plate with Vogel-Bonner E medium. Every concentration was tested without S9 fraction; with 4 % S9 fraction, and 10 % S9 fraction. The number of revertant colonies on the plates was recorded after 48 h of incubation in the dark at 37 °C. The background bacterial lawn was regularly checked by microscopy, as high doses of the extracts proved toxic to the tester strains, resulting in a thinning out of the background. According to the criteria given by Ames *et al.* [1], results were considered positive if the number of revertants on the plates containing the test concentrations was double that of the spontaneous reversion rate. The number of revertants per plate reported were determined from the linear part of dose-response curves, and the proportion to spontaneous reversion rates was calculated.

Results and discussion

In all experiments the filter extracts from RME exhaust induced lower mutation rates compared to DF, but there were distinct differences between the tested cycles and load modes. Because the transient tests (FTP-75, MVEG-A, MVEG-A1) are not completely comparable to the 5-mode test, the results are discussed separately.

Passenger car VW Vento 1.9l TDI

Diagram 12 presents the tester strain TA100 results from the VW Vento for different test cycles and graduated extract concentrations. Both the FTP-75 and the MVEG-A cycle indicate a comparable decrease of mutations when RME is used instead of DF. This approximately averaged 50 advantage for the alternative fuel is expressed more clearly in the cold start phase MVEG-A1, where the disadvantage for fossile petroleum diesel fuel is approximately averaged threefold versus RME.

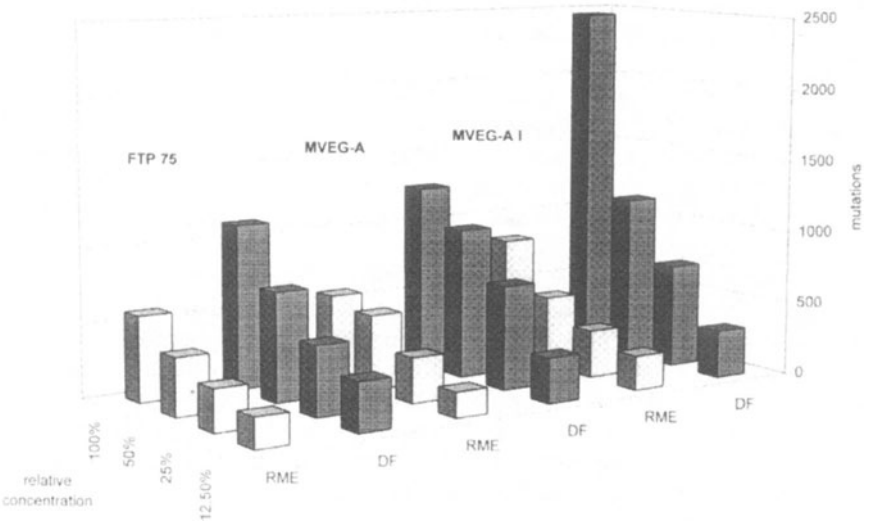

Diagram 12. Mutations caused by particulate extract from a passenger car on testerstrain TA100 (noise: 132 mutations/test)

In the tester strain TA98 similar elevated reversion rates were obtained. Testing with activated liver S9-fraction induced a distinctly lower increase of mutations. TA97a and TA102 showed no significant enhancement of spontaneous mutation rates.

Tractor Fendt Farmer 306 LSA

The results of the AMES-test with the strain TA100 are summarized in diagram 13. In all, the results indicate a higher mutagenic potency for DF than for RME, especially in the mode A and C. The tester strain TA98 gave similar results.

The tester strain TA97a showed no significant enhancement of spontaneous mutation rates. In TA102 inconsistently elevated rates were found, which showed no definite dose dependency. Therefore these results were not accepted as positive. The investigations at the tractor indicate in summary a higher mutagenic potency of DF compared to RME, although the emitted masses of RME were higher at 4 modes of the 5-mode test. Additionally, with both fuels, the mutagenic effects at rated power were higher than at running idle, as reported previously [26,27].

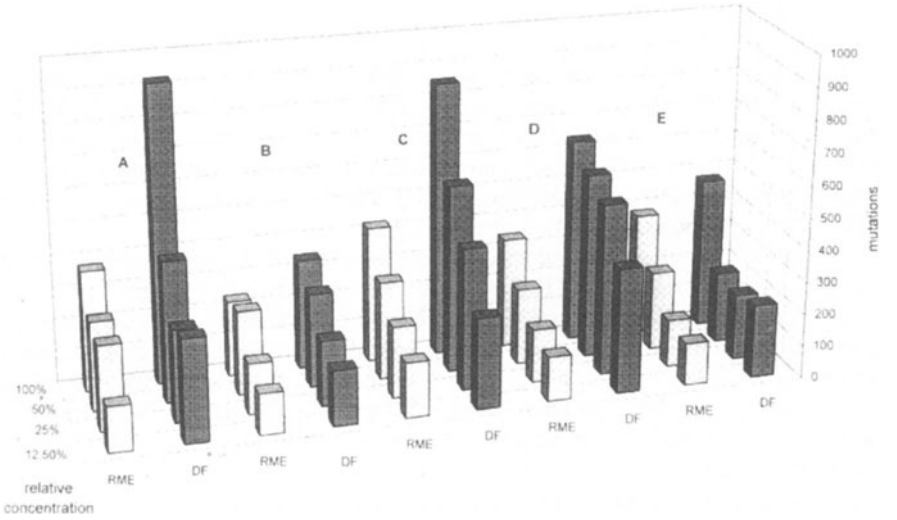

Diagram 13. Mutations caused by particulate extract from a tractor (noise: 126 mutations/test)

Present state of knowledge about genotoxic effects

Diesel engine emissions (DEE) act as mutagens in various *in vitro* test systems and are classified as probable carcinogenic agents to humans by the International Agency for Research on Cancer (IARC 1989) [11]. The mutagenic effects of particulate extracts of DEE from a passenger car and an agricultural tractor using RME as fuel were directly compared to DEE of fossile diesel fuel DF in the *Salmonella typhimurium*/mammalian microsome assay (AMES-test). In the tester strain TA100 a significant increase of spontaneous mutation rates was obtained for both fuels, but for DF revertants raised 2-6-fold compared to RME. In the tester strain TA98 similar elevated reversion rates were regarded. Testing with activated liver S9-fraction revealed markedly lower mutation rates.

In all, these results indicate a higher mutagenic potency of DEE of DF compared to RME. This is probably due to the lower content of PAH and soot, although the emitted masses of RME were higher in most cycles used in this study.

Conclusion

The comprehensive review of current research results shows both advantages and disadvantages of RME usage. It becomes obvious that by use of RME the mutagenic potency is significantly lower compared to DF. On the other hand, the aldehyde emissions of Biodiesel induce disadvantages for this alternative fuel. This publication, however, needs to expand on other specific characteristics of Biodiesel e.g. the size distribution of soot particles.

In light of the effects of emissions, current research at the German Federal Agricultural Research Centre (FAL) and at the University of Göttingen deals with a systematic investigation of the cytotoxic, mutagenic and carcinogenic characteristics and the particle size distribution of Biodiesel. Likewise, the ozone-forming potential of RME is going to be estimated in course of an actual research cooperation of the FAL and the Fraunhofer-Institute Hannover. Moreover, the goal of a future research must be a sound comparison of RME and SME. This may allow researchers to conclude that the DF/RME data base can be used when determining the influence of SME on environment and human health.

Summary

Based on the extensive literature about exhaust gas emissions resulting from the combustion of RME, data were compiled and differentiated according to the underlying engine test procedures, fuels and engine designs. In light of the known effects of individual exhaust gas components presented in the beginning, an effort was made to estimate the environmental benefits of RME compared to diesel fuel.

However, in order to make a sound evaluation of the emissions characteristics of Biodiesel, additional research is necessary. Likewise, the mutagenic, carcinogenic and cytotoxic effects resulting from RME use have not been sufficiently investigated when compared to the on-going investigation of these effects for petroleum diesel. In addition, in order to utilize Biodiesel in regions with high NO_x concentration, it is necessary to determine the expected ozone forming potential that results from the emissions and to compare it with that of petroleum diesel fuel.

References

1. B.N. Ames, J. McCann, E. Yamasaki, *Mutation Research* **31**, 347-364 (1975).
2. M.A. Belisario, V. Buonocore, E. De Marinis, F. De Lorenzo, *Mutation Research* **135**, 1-9 (1984).
3. C.R. Clark and C.L. Vigil, *Toxicology and Applied Pharmacology* **56**, 100-113 (1980).
4. L.D. Claxton, *Environmental Mutagenesis* **5**, 609-631 (1983).
5. G. Fränkle and H.-J. Stein, *Automobiltechn. Zeitschrift 90*, 13-22 and 85-92 (1988).
6. E. Garshick *et al.*, *American Review of Respiratory Diseases* **137**, 820-825 (1988).

7. P. Gustavsson, N. Plato, E.B. Lidstrom, C. Hogstedt, *Scandinavian Journal of Work and Environmental Health* **16**, 348-354 (1990).
8. A. Hartung, Personal Information, Volkswagen AG (1993).
9. U. Heinrich, Wirkungsforschung bei Dieselmotoremissionen. Proceedings: Emissionen von Pflanzenölkraftstoffen und ihre Umweltwirkungen. C.A.R.M.E.N.-Verlag, Würzburg (D), 19-29 (1994).
10. J. Huisingh *et al.*, Application of bioassay to the characterization of diesel particle emissions. In: Waters MD, Nesnow S, Huisingh JL, Sandhu SS, Claxton L (eds.) Application of short-term bioassay in the fractionation and analysis of complex environmental mixtures. Plenum Press, New York, pp. 383-418 (1978).
11. International Agency for Research on Cancer, Evaluation of carcinogenic risks to humans: Diesel and gasoline exhausts and some nitroarenes. *IARC Monographs*, **Vol. 46**, pp. 41-135 (1989).
12. I. Irmscher and N. Jaskulla, Aspekte des motorischen Verhaltens von Kraftstoffen aus pflanzlichen Ölen - Stand der internationalen Forschung. Final report: Verein für Innovative Verkehrstechnik, Berlin (D) (1994).
13. J. Krahl, G. Vellguth, A. Munack, Übersicht von Arbeiten zum Einsatz von Rapsöl und Rapsölmethylester als Kraftstoffe unter Berücksichtigung umweltrelevanter Auswirkungen. Final-report: Institute of Biosystems Engineering, FAL, Braunschweig (D) (1994).
14. J. Krahl, G. Vellguth, A. Munack, K. Stalder, M. Bahadir, *SAE Paper 961847* (1996).
15. C. Krausgrill and C. Schmidt, Abschlußbericht RME-Projekt Stadt Fulda. Final report: Technical University Darmstadt (D) (1993).
16. D.E. Levin, E. Yamasaki, B.N. Ames., *Mutation Res.* **94**, 315-330 (1982).
17. D.E. Levin, M. Hollstein, M.F. Christman, E.A. Schwiers, B.N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7445-7449 (1982).
18. D.M. Maron and B.N. Ames, *Mutation Research* **113**, 173-213 (1983).
19. T. Matsushima, M. Sawamura, K. Hara, T. Susimura, A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system. In: Serres FJ, Fouts JR, Bend JR, Philpot RM (eds) *In vitro* metabolic activation in mutagenesis testing. Elsevier/North-Holland, Amsterdam, pp. 85-88 (1976).
20. J.L. Mauderly, *Environmental Health Perspectives* **102 Suppl. 4**, 165-171 (1994).
21. J.E. Muscat, *Journal of clinical epidemiology* **49**, 891-892 (1996).
22. D.H. Pfluger and C.E. Minder, *Sozial- und Präventivmedizin* **39**, 372-378 (1994).
23. Sachverständigenrat für Umweltfragen, Umweltgutachten 1994. Kusterdingen, Metzler-Poeschel-Verlag (1994).
24. F. Schäfer, *Motortechn. Zeitschrift* **52**, 346-355 (1991).
25. K. Scharmer, H. Glüsing, L. Krings, Standardanforderungen an Rapsölmethylester als Motorenkraftstoff. Teil I: Stand der Technik. Report: Gesellschaft für Entwicklungstechnologie, Aldenhoven (D) (1993).
26. K. Stalder, V. Gerhard, H. Frohmann, J. Krahl, *Verhandlungen der Deutschen Gesellschaft für Arbeits- und Umweltmedizin* **33**, 75-78 (1993).
27. K. Stalder, V. Gerhard, J. Krahl, Zur kanzerogenen Wirkung von Rußen aus Dieselmotoren im Betrieb mit Rapsöl, RME und Dieselkraftstoff. In: Proceedings: Emissionen von Pflanzenölkraftstoffen und ihre Umweltwirkungen. C.A.R.M.E.N.-Verlag, Würzburg (D), pp. 30-35 (1994).
28. N.K. Steenland, D.T. Silverman, R.W. Hornung, *American Journal of Public Health* **80**, 670-674 (1990).

29. W. Stöber and U.R. Abel, *International Archives of Occupational and Environmental Health* **68** Suppl. 3-61 (1996).
30. G. Vellguth, *Grundl. Landtechnik* **32**, 17-186 (1982).
31. G. Vellguth, *Grundl. Landtechnik* **37**, 207-213 (1987).
32. G. Welschof, Der Ackerschlepper - Mittelpunkt der Landtechnik. VDI-Berichte 407, Düsseldorf (D), VDI-Verlag, pp. 11-17 (1981).
33. U. Wolfensberger, *Raps* **12(1)** 12-14 (1994).
34. F. Wurst, R. Boos, R. Prey, K. Scheidl, M. Wörgetter, Emissionen beim Einsatz von Rapsölmethylester an einem Prüfstandsmotor. Forschungsberichte der Bundesanstalt für Landtechnik, Vol. 22, Wieselburg (A) (1990).

The Mutagenic Potential of Diesel and Biodiesel Exhausts

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Abstract

There is a large body of literature on the association of mutagenicity and carcinogenicity with the use of fossil diesel fuel. For RME (methyl ester of rape oil), on the other hand, there are only a few data available on the mutagenicity of the particle fraction as determined by the Ames-test, which has indicated that RME exhausts are less mutagenic than diesel exhausts. Therefore, we collected samples of the particle and volatile fraction of both diesel and RME exhausts at different engine powers and evaluated the mutagenic potential in metabolically competent rat hepatocytes (endpoints tested: cytotoxicity, mitotic index, induction of micronuclei, chromosomal aberrations and sister chromatid exchanges). In parallel, we determined the mutagenic potential of these samples in the Ames-test (strains: TA 98 and TA 100).

Diesel and RME exhausts were compared in terms of: a) concentration dependence of the cyto- and genotoxic effects and b) mutagenic potential of particle extracts and condensates (volatile fraction) at a sample volume corresponding to one liter of exhaust gas. The results obtained differed with respect to the test system applied: While the results of the Ames-test point to a higher mutagenic potential of diesel exhausts, the hepatocyte assay results indicate both RME and diesel exhaust have high mutagenic potentials. In some cases, i.e. particle extracts collected at idle motion, diesel exhaust induced significantly elevated levels of chromosomal aberrations compared to RME. On the other hand, condensates of diesel exhausts collected at idle motion induced lower levels of chromosomal aberrations. These differences appear to be due to the different cytotoxic potential of the samples, which varied from sampling to sampling, but were generally more pronounced in diesel exhausts.

The potential health risk associated with the use of RME may be lower compared to diesel because diesel exhausts exhibit a higher cytotoxicity and they are more mutagenic in the Ames-test. However, the mutagenic potentials of RME and diesel exhaust in the hepatocyte assay are similar. Because particle emission is

significantly lower for RME, their particle associated carcinogenic potential may be lower than that of diesel exhaust.

Introduction

Recently, efforts have been made to obtain fuels from plant oils, particularly rapeseed oil, as an alternative to fossil diesel fuel. While the long-term operation of modern diesel engines with pure rapeseed oil is not possible, RME (methyl ester of rape oil) appears to be a useful fuel.

The use of fossil diesel fuel is associated with mutagenicity [1-9] and carcinogenicity - primarily induction of lung cancer - which is dependent on the species and even the strain of the same species used [10-13]. Additionally, epidemiological studies of exposed populations such as underground miners, diesel truck or bus drivers, heavy equipment workers and railroad workers [14-20] showed significant dose-response relationships between lung cancer induction and diesel exhaust exposure [16, 17], as well as an increased risk of bladder cancer [19]. In contrast, the studies by Raffle [14] and Wong [15] indicate a decreased lung cancer risk for traffic personnel and heavy equipment workers, while others failed to demonstrate a correlation between lung [21] and bladder cancer [22] and diesel exhaust exposure.

For RME, on the other hand, there are only a few data available on the mutagenicity of the particle fraction [23, 24] as determined by the Ames-test that has indicated that RME exhausts are less mutagenic than diesel exhausts. The lower mutagenic potential of RME compared to diesel exhausts is most pronounced with samples taken at idle motion.

Since the Ames-test requires an external metabolizing system (S9 liver microsomal fraction) to determine the mutagenicity of promutagens, i. e. benzo(a)pyrene, our research group focuses on the use of primary hepatocytes. Based upon their biotransformation capacity, which enables both the metabolic activation and detoxification of xenobiotic substances, primary cultures of adult rat hepatocytes have been shown to be highly sensitive and reliable indicators for the genotoxic potential of mutagens/promutagens [25, 26] and complex environmental mixtures [27-29]. Applying the hepatocyte assay to RME and diesel exhaust samples drawn at 50 % engine power and idle motion [30, 31], the mutagenic potential of the RME exhausts was not significantly different from that of the diesel exhausts, unlike in the Ames assay.

In this study we have further evaluated the mutagenic potential of diesel- and RME exhausts drawn at the same and additional engine powers (see table I) in order to assess the potential health risk associated with the use of RME and fossil diesel fuel under different conditions.

Materials and Methods

Material

MEM with Earle's salts and non-essential amino acids was obtained from Grand Island Biological Company through Life Technologies Overseas, Vienna, Austria. Plastic culture dishes were from Greiner, Kremsmünster, Austria. EGF, collagenase and other cell culture chemicals - unless otherwise specified - were purchased from Sigma Chemical Company, Munich, Germany. *Salmonella* tester strains were kindly provided by Dr. S. Knasmüller, Institute of Tumor Biology, University of Vienna.

Female Fischer 344 rats weighing approximately 100g were obtained from HARLAN, Zeist, The Netherlands. They were housed in hanging cages in a temperature (22±2°C)- and humidity (65±10 % relative humidity) controlled room with a 12 h light-dark cycle and fed with laboratory chow T783 from Tagger AG, Graz, Austria. Water was provided *ad libitum*. Sawdust served as bedding material and was obtained from Chemie Linz AG, Linz, Austria. The animals were allowed to acclimate for at least two weeks prior to hepatocyte isolation.

Methods

Exhaust sampling: Exhaust samples were drawn at the Bundesanstalt für Landtechnik at Wieselburg, Austria. The test engine was a tractor engine Steyr WD 411/87, which has also been used in previous experiments with RME [32]. A fraction of the emissions was isokinetically drawn through a stainless steel probe. The particles were collected on glass-fiber filters (Whatman), the volatile fractions (condensates) were precipitated at -196°C (liquid nitrogen).

Table I. Measuring points

measuring point	load (%)	revolutions per minute
1	100	2150
2	50	2150
3	100	1600
4	50	1600
5	0	900

The filters were further extracted with dichloromethane for 36 hours, concentrated by rotary evaporation, and the remaining solvent removed under a stream of nitrogen. The residues were dissolved in 10 ml of DMSO and stored in the dark at -20°C. The condensation trap was washed with 16 ml of dimethylsulfoxide (DMSO). DMSO and condensates were mixed and stored under the same conditions as the filters.

Ames-test

The Ames-test was carried out as 'plate incorporation assay' with the *Salmonella* tester strains TA 100, TA 98 and TA 98NR according to the protocol of Maron and Ames [33]: 0.1 ml of stationary phase cells were plated with various concentrations of the filter extracts and the condensates with and without S-9 mix (100 µl) and 3.0 ml of top agar on selective media plates. The plates were incubated for 48 hours, then the number of His^+ revertant colonies was counted. Three plates were enumerated per experimental point.

Hepatocyte assay

Hepatocytes were isolated from female Fischer 344 rats by the *in situ* two-step collagenase perfusion technique described by Michalopoulos et al. [34]. The isolated hepatocytes were plated at a density of 15 000 viable cells/cm^2 on collagen coated 60-mm diameter plastic culture dishes (Greiner). The hepatocytes were cultured in 5 ml of MEM supplemented with non-essential amino acids, pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM), gentamicin (50 µg/ml) and 0.4 mM calcium at 37 °C, 5 % CO_2 and 95 % relative humidity. After an incubation period of 3 hours the medium was exchanged for freshly prepared medium supplemented as described above with the further addition of insulin (10^{-7}M). After replacing the medium, the cultures were returned to the incubator. Approximately 20 hours after the initial exchange of the medium, the cultures were treated with the filter extracts and condensates for 3 hours. The fractions were applied directly to the cultures to a) yield final concentrations of 5 %, 1 %, 0.1 % and 0.01 % and in order to enable direct comparison (the exhaust volume collected was not exactly the same for all samples) to b) correspond to a sample volume of 1 dm^3. Benzo(a)pyrene (BAP), 1,8-dinitropyrene and 2-nitrofluorene served as positive controls, the solvent DMSO and untreated cultures as negative controls. After treatment the medium was aspirated, the plates were washed twice with fresh medium, the fresh medium was supplemented with EGF (40 ng/ml) and BrdU (10 µM), and the plates were returned to the incubator.

Forty-eight hours later, colcemid (0.4 µg/ml) was added and the cultures incubated for a further 3 hours. For SCE- and chromosomal aberration analysis, the medium was replaced with 2 ml of collagenase solution (0.5 mg/ml), and the plates returned to the incubator for an additional 10 min. The detached cells were collected by centrifugation, treated with hypotonic solution (0.01 M KCl) for 10 min and fixed in cold methanol-glacial acetic acid overnight. Preparations were made by dropping the cell suspension in fixative on precleaned glass slides followed by flame drying.

The slides were stained with Hoechst 33258 (4.5 µg/ml) for 15 min, rinsed with distilled water, mounted with a coverglass in PBS (pH=7) and exposed for 1 hour to a 40-W blacklight lamp (Philips TLD 36W08) on a warming plate kept at 50 °C. After removal of the coverglasses the slides were briefly washed with distilled water and stained in 5 % Giemsa solution. For determining chromosomal aberrations and SCE 20 well spread metaphases for each parameter were scored individually to obtain the mean±SD for a single experiment. The number of

aberrations is given per diploid cell, i.e. 42 chromosomes. The SCEs are reported per chromosome.

For the determination of the mitotic index and the number of cells with micronuclei, cultures not supplemented with colcemid were fixed with cold 3:1 methanol:glacial acetic acid for 5 min on the petri dishes, rinsed with distilled water for 2 min and air dried. The fixed cells were stained with DAPI (0.2 µg/ml McIlvaine citric acid - Na_2HPO_4 buffer, pH=7) for 30 min, washed with McIlvaine buffer for 2 min, briefly rinsed with distilled water and mounted in glycerol. To determine the mitotic index and the number of cells with micronuclei 1000 cells per petridish (2 dishes per concentration) were analyzed under a fluorescence microscope.

Statistics

Student's t-test for independent variables was used to calculate the levels of significance.

Results

Controls

The levels of genotoxicity found for the positive and negative controls are summarized in tables IIa and IIb.

Table IIa. Levels of revertant colonies induced by the negative and positive controls applied (mean ± SD of ≥3 independent experiments)

	revertants per plate				
	TA 100		TA 98		TA 98NR
control	- S9	+ S9	- S9	+ S9	
distilled water	128 ± 42	133 ± 11	24 ± 5	19 ± 9	56 ± 11
dimethylsulfoxide (DMSO, 1%)	115 ± 29	118 ± 14	17 ± 12	28 ± 22	
benzo(a)pyrene 10 µM	222 ± 71	605 ± 179*	54 ± 4	140 ± 23*	
1,8-dinitropyrene 0.7 nM			1441 ± 67*		1041 ± 37*
2-nitrofluorene [1] 1 nM			52 ± 3*		49 ± 1
3-nitrofluoranthene [1] 1 nM			358 ± 81*		63 ± 4

*: $P<0.05$; [1] :mean of two independent experiments

Table IIb. Levels of chromosomal aberrations induced by the negative and positive controls applied (mean ± SD of ≥3 independent experiments)

control	chromosomal aberrations	percentage of cells with micronuclei
distilled water	0.05 ± 0.03	5.3 ± 0.2
dimethylsulfoxide (DMSO, 1%)	0.06 ± 0.01	6.0 ± 1.0
benzo(a)pyrene 100 µM	0.39 ± 0.07*	6.6 ± 0.3
1,8-dinitropyrene 0.7 nM	0.10 ± 0.03	4.3 ± 0.7
2-nitrofluorene [1] 1 nM	0.04 ± 0.003	5.6 ± 1.2
3-nitrofluoranthene [1] 1 nM	0.07 ± 0.004	5.9 ± 1.4

*: P<0.05; [1] :mean of two independent experiments

The levels obtained with the *Salmonella* tester strains are comparable to those in the literature. The most characteristic differences observed between the Salmonella tester strains and primary rat hepatocytes were: a) the lack of genotoxicity of the nitro-PAHs at the concentration inducing significant levels of revertants in the Ames-test and b) the low capacity of B(a)P to induce clastogenic damage in hepatocytes. This observation is consistent with the findings of Müller et al. [35] and Eckl and Raffelsberger [36].

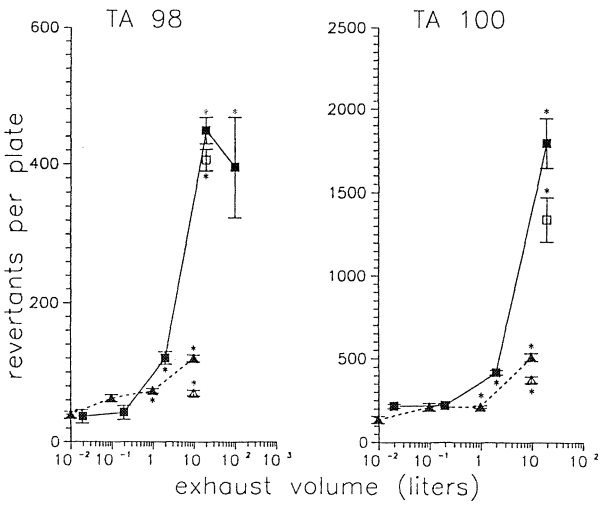

Figure 1. Mutagenicity of diesel (squares) and RME (triangles) exhaust particle extracts collected at idle motion (0 % load) in the Ames-test, *Salmonella* strains TA 100 and TA 98 (mean ± SEM of 3 independent experiments). Open symbols: with metabolic activation (S9); *: P<0.05.

Dose response experiments

In order to determine differences in the dose responses between the Ames-test and the hepatocyte assay, and to define an optimal sample volume to compare the mutagenic potentials of the exhaust samples, we investigated the dose response of the samples, in particular of the samples drawn at 0 % load (idle motion). This has been demonstrated previously to give the largest differences between diesel- and RME-exhaust particle extracts [23, 24].

As shown in figure 1 this difference holds true for the Ames-test with the strains TA 98 and TA 100. In the hepatocyte assay, on the other hand, a different dose response was observed. Both the particle extracts and the condensates (gaseous fraction) induced no significant dose response of micronucleus induction over this concentration range when the percentage of cells with micronuclei was corrected for cell proliferation (percentage of cells with micronuclei divided by the mitotic index; tables III, IV). However, both diesel- and RME-exhausts led to a clear dose response of chromosomal aberration induction (figures 2, 3) which was almost identical for condensate samples. The particle extracts differed with respect to the dose response: The diesel exhaust particle extracts led to an increasing number of chromosomal aberrations to a maximum level followed by a decrease with higher concentrations. At a concentration of 5 % the sample was found to be cytotoxic. RME exhaust particle extracts on the other hand were characterized by a very high standard deviation at 5 %. At this concentration no cell death was observed, however, there was a trend to lower mitotic indices (table IV).

Table III. Induction of micronuclei by diesel and RME exhausts drawn at idle motion (0 % load)

concentration	diesel exhaust		RME exhaust	
	particle extracts	condensates	particle extracts	condensates
control	4.8 ± 0.8	4.8 ± 0.8	4.6 ± 0.4	4.6 ± 0.4
0.01	3.6 ± 1.2	5.3 ± 0.9	4.0 ± 0.6	3.4 ± 0.8
0.1	4.0 ± 0.3	5.6 ± 0.2	4.0 ± 0.5	4.3 ± 0.6
1	2.7 ± 0.8*	4.7 ± 1.2	4.1 ± 0.7	3.5 ± 1.0
5	cytotoxic	cytotoxic	5.7 ± 0.9	cytotoxic

the corresponding air volumes at a concentration of 1% were: diesel exhaust particle extract: 9.8 l; diesel exhaust condensate: 2 l; RME exhaust particle extract: 6.9 l and RME exhaust condensate: 1.4 l
micronucleus data are standardized to a mitotic index of 1%
number of independent experiments: 3
*: $P<0.01$

Table IV. Influence on cell proliferation of diesel and RME exhausts drawn at idle motion (0 % load)

concentration	diesel exhaust		RME exhaust	
	particle extracts	condensates	particle extracts	condensates
control	2.4 ± 0.3	2.4 ± 0.3	2.8 ± 0.3	2.8 ± 0.3
0.01	2.8 ± 0.9	2.4 ± 0.4	4.1 ± 0.6*	2.8 ± 0.5
0.1	2.7 ± 0.6	2.2 ± 0.2	3.9 ± 0.2**	3.4 ± 0.2+
1	2.4 ± 0.5	3.1 ± 0.8	3.7 ± 0.5+	4.7 ± 0.8*
5	cytotoxic	cytotoxic	2.7 ± 0.4	cytotoxic

the corresponding air volumes at a concentration of 1% were: diesel exhaust particle extract: 9.8 l; diesel exhaust condensates: 2 l; RME exhaust particle extracts: 6.9 l and RME exhaust condensates: 1.4 l
number of independent experiments: 3
+: P<0.05; *: P<0.01; **: P<0.001

Mutagenic potential at an exhaust volume of 1 liter

The results of the experiments carried out with the Salmonella strains TA 100 and TA 98 and the hepatocyte assay with a sample volume corresponding to an exhaust volume of one liter are summarized in figures 4-9 and table V (TA 98NR).

Table V. Mutagenicity of diesel and RME exhausts at an exhaust volume of 1 liter (Salmonella test strain TA 98NR)

measuring points	particle extracts		condensates	
	diesel	RME	diesel	RME
control	56.3 ± 11.0	56.3 ± 11.0	56.3 ± 11.0	56.3 ± 11.0
1	49.3 ± 5.6	33.3 ± 8.7+	78.7 ± 11.4	45.7 ± 6.4
2	54.0 ± 7.8	38.7 ± 11.0	80.7 ± 7.8+	59.7 ± 4.6
3	49.0 ± 6.5	40.7 ± 16.7	25.0 ± 5.6+	30.7 ± 2.1
4	50.0 ± 6.2	27.3 ± 8.5+	62.0 ± 18.1	51.3 ± 17.5
5	80.3 ± 14.6	29.3 ± 4.7+	85.7 ± 8.4+	41.0 ± 12.2

+: P < 0.05

Ames-test

Strain TA 100 (figures 4,5): Significantly elevated numbers of revertants were induced by the diesel exhaust particle extracts (measuring points 1, 2, 3 and 5), the RME exhaust particle extracts (measuring points 3, 4 and 5), the diesel exhaust condensates (measuring points 1, 2, 4, and 5) and the RME exhaust condensates (measuring points 1, 3 and 4). Diesel exhaust particle extracts (measuring points 2, 3 and 5) and the condensates (measuring points 4 and 5) were significantly elevated compared to the corresponding RME samples.

Strain TA 98 (figures 6,7): Significantly elevated numbers of revertants were induced by the diesel exhaust particle extracts (measuring points 1, 3 and 5), the particle extract RME (measuring point 3), by the diesel condensates (measuring points 2, 4 and 5) and the RME exhaust condensates (measuring points 3, 4 and 5).

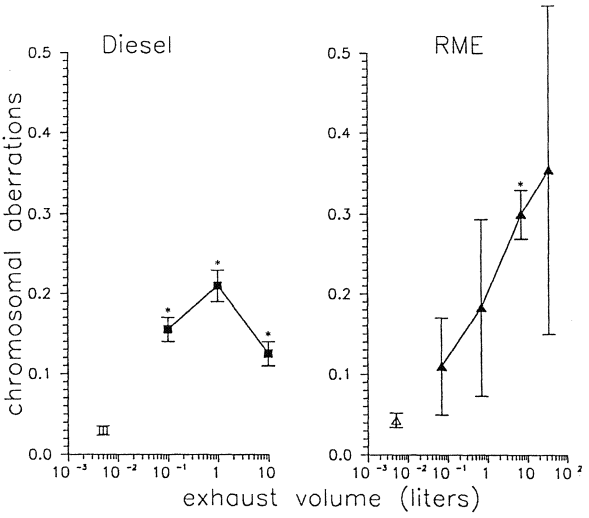

Figure 2. Induction of chromosomal aberrations by diesel (squares) and RME (triangles) exhaust particle extracts (mean ± SEM of ≥3 independent experiments). Open symbols: control level; *: P<0.05.

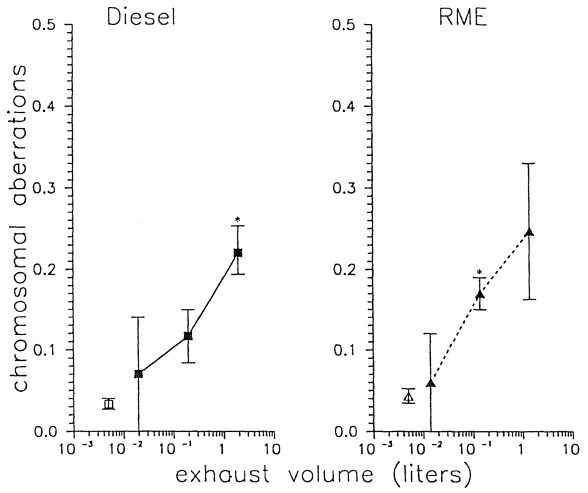

Figure 3. Induction of chromosomal aberrations by diesel (squares) and RME (triangles) exhaust condensates (mean ± SEM of ≥3 independent experiments). Open symbols: control level; *: P<0.05.

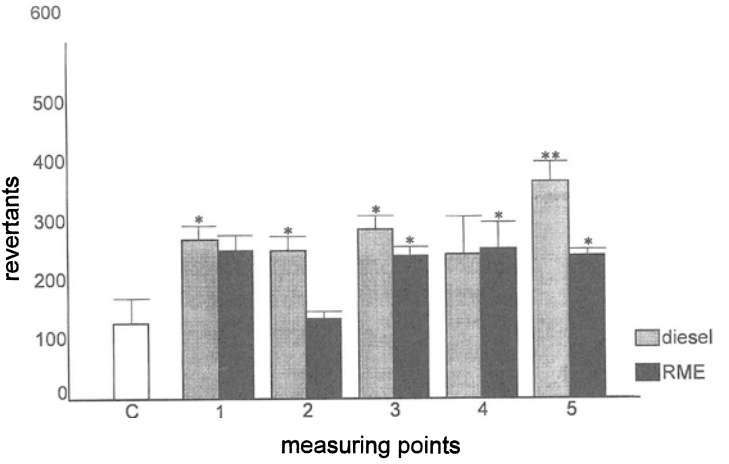

Figure 4. Mutagenicity of diesel and RME exhaust particle extracts at an exhaust volume of 1 liter (*Salmonella* test-strain TA 100; mean ± SEM of ≥3 independent experiments). *: P<0.05, **: P<0.01.

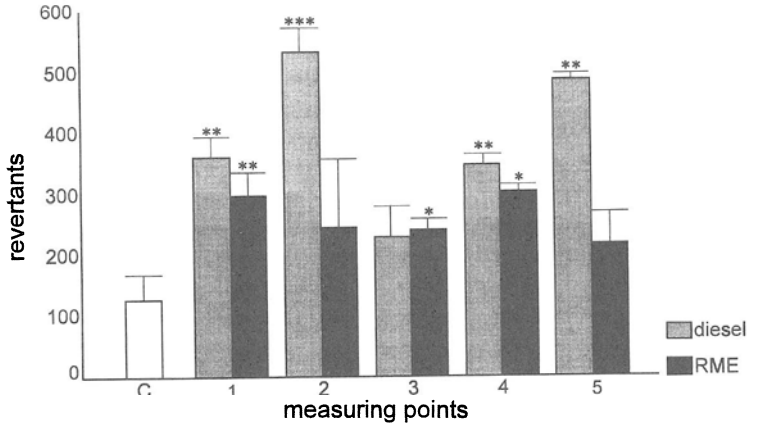

Figure 5. Mutagenicity of diesel and RME exhaust condensates at an exhaust volume of 1 liter (*Salmonella* test-strain TA 100; mean ± SEM of ≥3 independent experiments). *: P<0.05, **: P<0.01, ***: P<0.001.

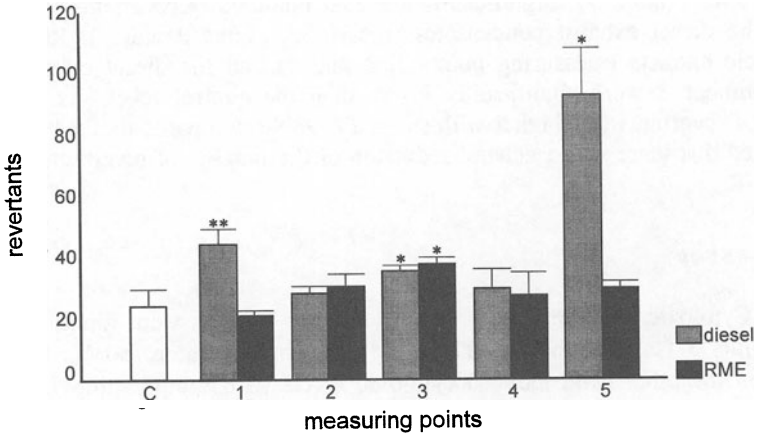

Figure 6. Mutagenicity of diesel and RME exhaust particle extracts at an exhaust volume of 1 liter (*Salmonella* test-strain TA 98; mean ± SEM of ≥3 independent experiments). *: P<0.05, **: P<0.01

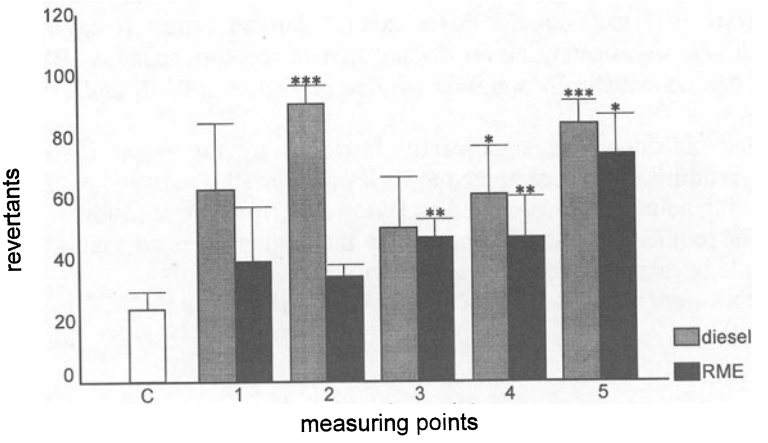

Figure 7. Mutagenicity of diesel and RME exhaust condensates at an exhaust volume of 1 liter (*Salmonella* test-strain TA 98; mean ± SEM of ≥3 independent experiments). *: P<0.05, **: P<0.01, ***: P<0.001.

Diesel exhaust particle extracts (measuring points 1 and 5), and the condensate (measuring point 2), were significantly elevated compared to the corresponding RME samples. For all other samples there was no significant difference between diesel and RME exhausts. With both Salmonella strains a similar or higher mutagenicity of the condensates was observed.

Strain TA 98NR (table V): Significantly elevated numbers of revertants were induced by the diesel exhaust condensates (measuring points 2 and 5). RME exhaust particle extracts (measuring points 1, 4 and 5) and the diesel exhaust condensate number 3 were significantly lower than the control level. As the control level of revertants was higher with strain TA 98NR compared to TA 98 it can be assumed that there was a general reduction of the number of revertants in strain TA 98NR.

Hepatocyte assay

Cytotoxicity: Cytotoxic levels of diesel exhaust particle extracts were found for measuring points 3 (sample volume: 12 l) and 5 (sample volume: 50 l). The condensates on the other hand induced cytotoxic levels with samples drawn at measuring points 2 (sample volume: 4 l), 3 (sample volume: 2.5 l) and 5 (sample volume: 10 l). Cytotoxic levels of RME exhausts were found solely with the condensate sample drawn at measuring point 5 (sample volume: 6 l).

Induction of chromosomal aberrations (figures 8, 9): Significantly elevated levels of chromosomal aberrations were determined for the diesel exhaust particle extracts (measuring points 2 and 5), and for the condensates (measuring points 3 and 4). On the other hand the RME exhaust particle extract (measuring point 3), and the condensates (measuring points 1, 4 and 5) induced significantly elevated levels of aberrations. Except for the diesel exhaust particle extract (measuring point 5) which was significantly elevated compared to the corresponding RME sample, there was no statistically significant difference between RME and diesel exhausts.

Micronucleus induction was significantly increased by the diesel exhaust condensate (measuring point 3) compared to RME, and the RME exhaust particle extract (measuring point 1) compared to diesel. However, when these results were corrected for the proliferation rate as measured by the mitotic index no significant differences could be demonstrated (data not shown).

No differences were seen between diesel and RME exhausts in the SCE assay (data not shown).

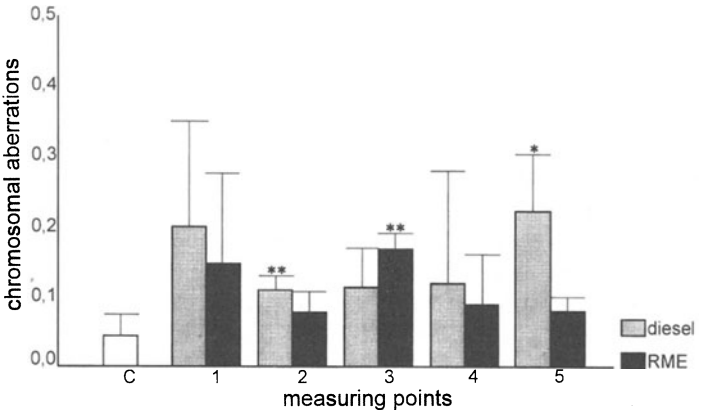

Figure 8. Induction of chromosomal aberrations in primary rat hepatocytes by diesel and RME exhaust particle extracts (mean ± SEM of ≥3 independent experiments). *: P<0.05, **: P<0.01.

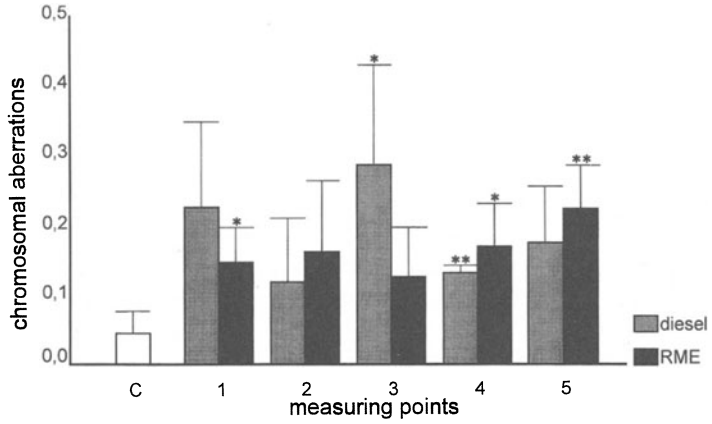

Figure 9. Induction of chromosomal aberrations in primary rat hepatocytes by diesel and RME exhaust condensates (mean ± SEM of ≥3 independent experiments). *: P<0.05, **: P<0.01.

Discussion

General comments

Induction of micronuclei

Micronuclei are the result of either chromosome breaks or disturbances of the mitotic spindle. Since their appearance requires cell division, the frequency of cells with micronuclei will increase with increasing proliferation rate although the level of damage remains the same. For example, the RME particle extract tested at a concentration of 0.01 % yielded 16.3 % cells with micronuclei and a mitotic index of 4.2 % compared to 12.8 % cells with micronuclei and a mitotic index of 2.8 % in the untreated controls. When the percentage of cells with micronuclei is standardized to a mitotic index of 1 % there is no more difference detectable (see table III). This mathematical transformation is of limited use when the cytotoxicity of a sample leads to very low mitotic indices. Therefore, the cytochalasin B method was developed to score only those micronuclei which definitely went through mitosis [37]. Cytochalasin B inhibits the formation of daughter cells leading to binucleated cells, and micronuclei are determined in the binucleated cells. Unfortunately, approximately 30 % of primary hepatocyte collectives are binucleated and the percentage of binucleated hepatocytes changes during primary culture [38]. To circumvent this limitation we developed a micronucleus assay which allows the determination of those micronuclei which underwent DNA replication after treatment [36], and which is based on immunocytochemical localization of BrdU and counterstaining with DAPI. This method, however, has not yet been applied to the exhaust samples.

Induction of chromosomal aberrations

The dose responses of chromosomal aberration induction obtained with the particle extracts may be attributable to an increased cytotoxic effect as previously discussed for water samples [28, 29]. In the first case - increase followed by a decrease - the toxic component appears to become prominent at the highest concentration, i.e. all cells are killed at a concentration of 5 % of the diesel exhaust particle extracts. It is therefore assumed that this cytotoxic effect may affect the cell population entering mitosis already at a concentration of 1 %. Under this assumption preferably the less damaged cells undergo mitosis thus reducing the number of chromosomal aberrations. In the case of RME exhaust particle extracts no cell killing is observed even at a concentration of 5 %. Therefore, the high standard deviation at this concentration may either reflect a less pronounced cytotoxicity or may be due to interindividual differences (for each experiment cells from a different animal were used) and different compositions of the

exhausts (the figure represents the mean of the experimental data obtained from two independently drawn samples).

Differences between the Ames test and the hepatocyte assay

The results of the Ames-test point to a higher mutagenic potential of the diesel exhausts compared to RME while the results obtained in the hepatocyte assay do not support these findings. This difference most probably has to be attributed to the different metabolic capacities. Primary hepatocytes are metabolically competent and can therefore activate promutagens or detoxify mutagens. Since the enzymes for the metabolism of xenobiotic substances are almost completely missing in Salmonella these are supplied as liver microsomal fractions (S9) in order to enable biotransformation [39]. However, even under these conditions false positive or false negative results may be obtained due to the loss or at least a change of the composition of the metabolic enzymes [40]. Therefore, it can be assumed that the results obtained in the Ames test may be of lesser relevance.

Specific discussion

An important finding is the observation that the condensates in general are more mutagenic than the filter extracts. This observation is also confirmed by the results of the hepatocyte assay, particularly by the results of the chromosomal aberration analysis. As previous investigations were carried out solely with particle extracts more attention has to be drawn to the characterization (chemical composition and potential health risk) of the volatile fractions of airborne pollutants.

As already mentioned, the results of the Ames-test point to a higher mutagenic potential of the diesel exhausts compared to RME.

Diesel exhaust particle extracts led to significantly higher levels of chromosomal aberrations compared to RME only at idle motion (0 % load). This result indicates a similar mutagenic potential of RME and diesel exhausts. Taking into consideration the cytotoxic potential which was more expressed by the application of diesel exhausts the relative health risk of RME can be considered to be lower compared to diesel exhausts. Additionally, particle emission has to be taken into consideration as particles are carcinogenic per se [41, 42]. Rape oil and especially RME lead to a reduced emission of particles compared to diesel [43]. Depending on the motor type a reduction in particles of up to 40 % can be observed [44]. Therefore, even if a higher mutagenic potential of RME exhausts could be confirmed for higher sample volumes at a particular measuring point, it can be concluded that the health risk associated with the use of RME is lower compared to that of diesel.

The differences between the results obtained in the Ames-test and the hepatocyte assay appear to be due to the sensitivity of the bacteria towards nitro-PAHs as evidenced with the Salmonella strains TA 98 and TA 98NR. The latter strain, which is nitroreductase deficient, led to a reduction of revertant colonies indicating the presence of mono-nitrated PAHs, which was further demonstrated

with the positive controls 2-nitrofluorene and 1,8-dinitropyrene (table IIa). When tested in the hepatocyte assay (table IIb), however, both compounds did not induce increases of chromosomal aberrations or micronuclei, even at concentrations up to 100 nM (data not shown) possibly indicating that nitro-PAHs may play a minor role for the the human health risk associated with automobile, particularly diesel and RME exhausts.

In order to correlate the observed mutagenicity with specific components of the exhausts we analyzed some of the suspect chemicals, in particular PAHs such as benzo(a)pyrene (data not shown). Additionally, aldehydes such as acrolein, and benzol, toluol and xylol were quantified. Applying correlation analysis (SPSS for Windows, bivariate correlation), however, no significant correlation between the measured components and the observed mutagenic potential was found. This observation is also confirmed when the mutagenic effect of particular components is compared with the concentrations determined for RME and diesel exhausts. For example, the mean concentration of benzo(a)pyrene in the exhausts collected at idle motion (measuring point 5) was determined to be 35 ng/m^3 for RME and 215 ng/m^3 for diesel. When applying the sample equivalent of 1 liter to the cultures (5 ml of medium) this relates to concentrations of 27 and 170 pM, respectively. Since B(a)P induces significant levels of chromosomal aberrations in hepatocytes in the micromolar range, B(a)P also appears not to be responsible for the mutagenic potential observed. Further investigations will have to be carried out to clarify the role of PAHs on the mutagenic potential of diesel and RME exhausts.

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References

1. T.C. Pedersen and J.-S. Siak J.-S., *J. Appl. Toxicol.* **1**, 54 (1981).
2. D. Schuetzle, F. S. C. Lee, T. J. Prater and S. B. Tejada, *Int. J. Environ. Anal. Chem.* **9**, 93 (1981).
3. A. P. Li, *Toxicol. Appl. Pharmacol.* **75**, 55 (1981).
4. A. P. Li and R. E. Royer, *Mutation Res.* **103**, 349 (1983).
5. W. G. Thilly, H. Longwell, and B. M. Andon, *Environ. Health Perspect.* **48**, 129 (1983).
6. R. O. McClellan, *Ann. Rev. Pharmacol. Toxicol.* **27**, 279 (1987).
7. R. E. Rasmussen, *J. Air Waste Manage. Assoc.* **40**, 1391 (1990).
8. T. Karube, Y. W. Kim, K. Kawamura, Y. Odagiri, H. Katayama and K. Takemoto, *Nippon Eiseigaku. Zasshi.* **46**, 646 (1991).
9. J. Song and S. H. Ye, *Biomed. Environ. Sci.* **8**, 240 (1995).

10. N. Ishinishi, A. Koizumi, R. O. McClellan and W. Stoeber, *Carcinogenicity and mutagenicity of diesel engine exhaust* (Elsevier, Amsterdam, 1986).
11. F. T. Cross, R. K. Palmer, R. E. Pilipy, R. H. Busch and B. O. Stuart, *Pac. Northwest Lab. Rep.* 2744 (1978).
12. H. White, J. J. Vostal, H. L. Kaplan and W. F. MacKenzie, *J. Appl. Toxicol.* **3**, 332 (1983).
13. L. Mauderly, D. A. Banas, W. C. Griffith, F. F. Hahn, R. F. Henderson and R. O. McClellan, *Fundam. Appl. Toxicol.*, **30**, 233 (1996).
14. P. Raffle *Br. J. Ind. Med.* **14**, 73 (1975).
15. O. Wong, R. W. Morgan, L. Keifets, S. R. Larson and M. D. Whorton, *Br. J. Ind. Med.* **42**, 435 (1985).
16. G. R. Howe, D. Fraser, J. Lindsay, B. Presnal and S. Z. Yu S.Z. *J. Natl. Cancer Inst.* **70**, 1015 (1983).
17. M. B. Schenker, T. Smith, A. Munos, S. Woske and F. E. Speizer, *Br. J. Ind. Med.* **41**, 320 (1984).
18. E. Garshick, A. Munoz, M. B. Schenker, S. Woskie, T. Smith and F. E. Speizer, *Am. Rev. Respir. Dis.* **133**, A264 (1986).
19. D. T. Silverman, R. N. Hoover, T. J. Mason. and G. M. Swanson, *Cancer Res.* **46**, 2113 (1986).
20. P. Gustavsson, N. Plato, E. B. Lidstrom and C. Hogstedt, *Scand. J. Work Environ. Health* **16**, 348 (1990).
21. J. E. Muscat and E. L. Wynder, *Environ. Health Perspect.* **103**, 812 (1995).
22. V. Iyer, R. E. Harris and E. L. Wynder, *Eur. J. Epidemiol.* **6**, 49 (1990).
23. K. Stalder, V. Gerhard, J. Krahl and A. Munack, *Landtechnik* **5**, 266 (1995).
24. K. Stalder, V. Gerhard and J. Krahl J., *Emissionen von Pflanzenölkraftstoffen und ihre Umweltwirkungen*, Rimpar bei Würzburg, (CARMEN e.V., Rimpar bei Würzburg) pp. 30-35 (1994).
25. P. M. Eckl, S. C. Strom, G. Michalopoulos and R. L. Jirtle), *Carcinogenesis* **8**, 1077 (1987).
26. T. Alati, P. Eckl and R. L. Jirtle, *Rad. Res.* **119**, 565 (1989).
27. P. M. Eckl, T. Alati and R. L. Jirtle, *Carcinogenesis* **12**, 643 (1991).
28. P. M. Eckl, L. Anderson-Carnahan and R. L. Jirtle, *Sci. Total Environ.* **135**, 111 (1993).
29. P. M. Eckl, *Sci. Total Environ.* **159**, 81 (1995).
30. W. Stöß, Thesis, University of Salzburg, Austria (1991)
31. P. Eckl and W. Stöß, in (24), pp. 109-119 (1994).
32. F. Wurst, R. Boos, T. Prey, K. Scheidl and M. Wörgetter, *Forschungsberichte der Bundesanstalt für Landtechnik, Wieselburg* **22** (1990).
33. D. M. Maron and B. M. Ames, in *Handbook of Mutagenicity Test Procedures*, B. J. Kilbey, M. Legator, W. Nichols, C. Ramel, Eds. (Elsevier, Amsterdam, 1983), pp. 93-141.
34. G. Michalopoulos, D. Cianciulli, A. R. Novotny, A. D. Kligerman, S. C. Strom and R. L. Jirtle, *Cancer Res.* **42**, 4673 (1982).
35. K. Müller, P. Kasper and L. Müller, *Mutation Res.* **292**, 213 (1993).
36. P. M. Eckl and I. Raffelsberger, *Mutation Res.*, in press.
37. M. Fenech, Mutation Res. 285, 35 (1993).
38. P. M. Eckl, J. Cell. Physiol. 154, 601 (1993).
39. Y. Y. Wang, S. M. Rappaport, R. F. Sawyer, R. E. Talcott and E. T. Wie, Cancer Lett. 5, 39 (1978).

40. S. DeFlora, Nature 271, 455 (1978).
41. U. Heinrich, F. Pott and S. Rittinghausen, *Carcinogenic and mutagenic effects of diesel engine exhaust* (Developments in Toxicology and Environmental Science), Ishinishi, N. et al., Eds. (Elsevier, Amsterdam, New York, Oxford, 1986), pp. 441-457.
42. U. Heinrich, in (24), pp. 19-29 (1994).
43. H. Richter, in (24), pp.36-46 (1994).
44. B. Widmann, in (24), pp. 72-93 (1994).

Biodiesel: Effects on Exhaust Constituents

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Abstract

Fatty-acid mono-ester fuels, commonly referred to as „Biodiesel" fuels, are currently under evaluation for use as an alternative diesel fuel in the United States. Blends of Biodiesel fuels with petroleum diesel fuel have been tested in a large number of urban bus transit demonstrations, and they are currently under consideration for commercial use in confined environments such as underground mines in the United States and Canada. The use of Biodiesel fuels, such as soy- and rapeseed-oil methyl esters, has been shown to significantly reduce emissions of the carbon-soot constituents of diesel particulate matter (PM), while increasing the emissions of soluble or volatile organic particulate matter. The use of Biodiesel fuels has also been shown to reduce the emissions of some PAH and nitro-PAH compounds. When used in conjunction with an appropriately selected diesel oxidation catalyst, significant reductions in total PM emissions from diesel engines have been realized. This paper will present a summary of recent work conducted in the United States to characterize the exhaust emissions of diesel engines using a soy-methyl-ester Biodiesel fuel, including the effects of the Biodiesel fuel on exhaust gas composition, PM composition, and particle-size distribution.

Introduction

Under contracts with the Federal Government of the United States[2], the University of Minnesota - Center for Diesel Research (CDR) is engaged in a program of research to reduce exposure to particulate matter (PM) from diesel exhaust emissions in underground mines. The U.S. Mine Safety and Health Administration (MSHA) has announced the intent to limit exposure to PM

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[2]Contract number USDI-1432 C0369004.

emissions from diesel engines [1]. In 1995, the American Conference of Governmental Industrial Hygienists added diesel exhaust to its notice of intended changes [2], designating it as a suspected human carcinogen, and recommending a threshold limit value (TLV^{TM}) of 0.15 mg/m^3.

Control of PM emissions from diesel equipment has been emphasized because it is a major component of diesel exhaust that is a suspected health hazard due its associated adsorbed and/or condensed polynuclear aromatic hydrocarbon (PAH) compounds and its respirable, primarily submicron particle-size [3]. PM concentrations from diesel exhaust in underground coal, metal, and nonmetal mines in North America typically range from 0.2 to 1.0 mg/m^3 [3, 4, 5, 6], although instances significantly exceeding 1 mg/m^3 have been reported [5, 6].

PM emissions from diesel engines are complex mixtures of solid, carbon soot, adsorbed and condensed organic material (including PAH and other organic material), condensed sulfuric acid, water, ash, metals, and other trace materials. The particle-size distribution of this aerosol mixture in diesel exhaust is approximately bimodal, with a nuclei mode of particles having aerodynamic diameters of approximately 0.002 to 0.03 μm, and an accumulation mode of agglomerated particles with aerodynamic diameters of approximately 0.01 to 1.0 μm. The mass-mean diameter of the accumulation-mode particles is typically in the range of 0.2 to 0.3 μm, and is characterized by an approximately log-normal distribution of particle-size. A high degree of particle deposition occurs within the pulmonary region of human lungs for fine particles with aerodynamic diameters of less than approximately 0.2 μm [7]. An idealized schematic showing some of the typical chemical and physical characteristics of PM emissions from diesel engines is presented in figure 1.

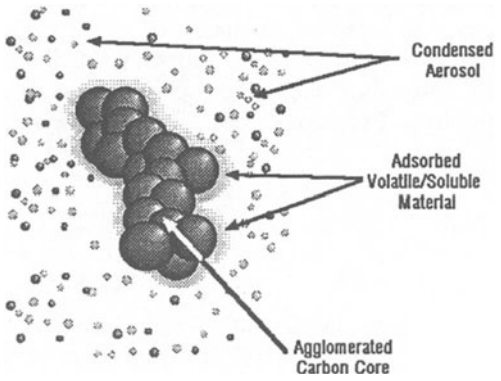

Figure 1. Schematic of PM in diesel exhaust. The diameter of the individal spherols in the carbon-soot aggregate is approximately 30 nm.

Oxygenated diesel fuels are one approach for reducing combustion formation of carbon soot constituents that contribute to PM emissions [8, 9, 10]. The use of such passive controls as oxygenated alternative diesel fuels or fuel additives is

particularly attractive for older, existing diesel equipment since few changes to the equipment or refueling infrastructure are needed.

Fatty-acid mono-ester fuels, commonly known as Biodiesel fuels, contain approximately 11 % oxygen by mass. As with other oxygenated diesel fuels, Biodiesel reduces carbon soot emissions by increasing oxygen availability to locally fuel-rich regions within the cylinder during combustion. This increase in oxygen availability may reduce the pyrolysis of unburned or partially burned fuel compounds that is believed to be the primary mechanism of carbon-soot formation during diesel combustion [11, 12]. Unlike other oxygenated fuels, Biodiesel fuels typically increase the formation of particle-phase volatile organic compounds (PM-VOC), also referred to as the soluble organic fraction of diesel particulate matter (PM-SOF) [13, 14, 15]. Similar to other oxygenated diesel fuels, a general trend towards somewhat higher emissions of oxides of nitrogen (NO_x) have been reported [8, 9, 14, 15], but not in all cases [13].

In order to evaluate the effectiveness of the use of Biodiesel fuels as potential PM emissions controls for diesel engines operated in confined spaces, the following project goals were identified:

1. Identify and characterize exhaust emissions with potential health effects, including:
   - quantification of PAH and n-PAH emissions with Biodiesel fuel
   - determination of effects of the fuel on exhaust mutagenic activity
   - determination of the effects of the fuel on PM size
2. Determine the source or sources of the high PM-SOF emissions characteristic of Biodiesel fuel use;
3. Investigate NO, NO_2, and total NO_x emissions and analyze engine to engine variations in these emissions during operation on Biodiesel fuel.

This research program began under the auspices of the Diesel Technology Group of the U.S. Bureau of Mines in 1993. The program transferred, along with the researchers and laboratory facilities of the Diesel Technology Group, to the University of Minnesota upon the formation of the Center for Diesel Research and the closure of the U.S. Bureau of Mines in early 1996. The information offered for these proceedings is a summary of research that was, in part, previously published or in publication in three separate papers in the United States [16, 17, 18].

Experimental Methods

The first diesel engine tested was a 7-liter, 4-cylinder, indirect injection (IDI), pre-chamber, naturally aspirated, Caterpillar 3304 PCNA. Maximum power output was 75 kW @ 2200 rpm. This particular engine was chosen because of its wide use within the underground mining industry. The 3300 series of Caterpillar engines are used in loading and haulage equipment in underground metal, nonmetal, coal mines, and also in other industrial applications. The Caterpillar engine was tested both with and without a modern, low sulfate forming diesel oxidation catalyst (DOC) [16, 17]. DOCs are diesel-specific catalytic converters that are commonly used in underground metal and nonmetal mine applications for

the control of exhaust odor and CO emissions. Modern DOC formulations have also proven effective at reducing PM-SOF emissions with only minimal formation of sulfate-PM. The engine was equipped with piezo-electric cylinder pressure and fuel pressure transducers, and an optical crankshaft-position encoder for measurement of cylinder pressure and fuel injection rate versus crankshaft position. Apparent rate of heat release and other engine-cycle thermodynamic properties were calculated using the methods of Krieger and Borman [19].

The second engine tested was a Deutz-MWM D916-6, 6.2 liter, IDI, swirl-chamber, naturally aspirated engine. The maximum power output of the engine was 70 kW @ 2500 rpm. This engine is commonly used in coal mine haulage equipment and in other industrial and marine applications. Engine testing was conducted in two separate, U.S. E.P.A.-style engine test cells utilizing eddy-current dynamometers. Complete descriptions of the engine test cells can be found in Anderson *et al.*, McDonald *et al.* and Purcell *et al.* [16, 17,20].

The fuels evaluated included a baseline fuel consisting of a commercially available, U.S. low-sulfur number 2 diesel fuel (D2) and a 100 % soy methyl ester Biodiesel fuel (transient and steady-state testing). The Caterpillar engine was additionally tested using a 30 % (by mass) blend of Biodiesel with 70 % D2 (B30) (steady-state testing only) as a lower cost alternative to Biodiesel. Fuel characteristics are summarized in tables 1 and 2.

Both steady-state and transient test-cycles were used for the emissions evaluations. The Caterpillar and MWM engines were both tested using the ISO 8178-C1 8-mode steady-state test-cycle. This cycle has been adopted both in the U.S. and Europe as the standard test-cycle for certification and approval of the exhaust emissions of off-road diesel engines [21, 22]. This test cycle was selected in part because of its wide-spread usage for certification, research, and development. This would allow general comparisons of results from the combinations of engines and fuels used in this research program to other results in the literature over the same standardized engine operating cycle. The measurement of particle size from diesel exhaust also required operation over a steady-state speed-load test cycle to allow sufficient time for the particle-size instrumentation to measure particle concentrations versus intervals of particle-size.

The Caterpillar engine was additionally tested using two transient test-cycles developed by the U.S. Bureau of Mines (USBM) for the evaluation of exhaust emissions controls for diesel powered production equipment used in underground mines. The test-cycles are described in detail in McDonald *et al.*, Purcell *et al.*, and McClure *et al.* [16, 17, 23]. The USBM light-duty transient test-cycle had an average load factor of approximately 20 %. This test-cycle included a number of high-speed, light-load operating conditions that often result in high emissions of PM-SOF and engine-out NO_2[3].

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[3] Engine-out is defined as upstream of any exhaust aftertreatment device, or with the absence of such device. Many of these devices oxidize NO to NO_2. Light-load conditions tend to increase NO_2 emissions when no exhaust aftertreatment is used. Conversely, heavy-load, high exhaust temperature conditions increase oxidation to NO_2 when some exhaust aftertreatment devices are used.

Table 1. Fuel Properties (Caterpillar Engine Evaluation)[a]

Properties	D2	Biodiesel	B30	
Cetane Number (ASTM D287)	43.2	54.7	49.1	
Lower Heating Value, MJ/kg (ASTM D240)	42.8	37.1	40.5	
C:H:0 Molar Ratio	16:30:0	19:34:2		17:31:0.5
Sulfur, Mass %	0.010	<0.005		<0.005
Oxygen, Mass %	0	11.0	3.4	
Aromatics, Mass %	39.0	0.0		27.3
Kinematic Viscosity, cSt @ 40 °C	2.37	3.05		2.6
API Gravity	33.2	28.3	31.6	
Cloud Point, °C (ASTM D2500)	-21	-2	-17	
Flashpoint, °C (ASTM D93)	68	160	77	

[a] D2: US low-sulfur No. 2 petroleum diesel fuel; Biodiesel: fatty-acid methyl-esters of soy oil; B30: 30 % Biodiesel/70 % D2 (by mass).

Table 2. Fuel Properties (MWM Engine Evaluation) [a]

Properties	D2	Biodiesel	
Cetane Number (ASTM D287)	43.2	55.0	
Lower Heating Value, MJ/kg (ASTM D240)	42.8	37.1	
C:H:0 Molar Ratio	16:30:0	19:34:2	
Sulfur, Mass %	0.018	<0.005	
Oxygen, Mass %	0	11.0	
Aromatics, Mass %	26.0	0.0	
Kinematic Viscosity, cSt @ 40 °C	3.12	4.2	
API Gravity	34.1	28	
Cloud Point, °C (ASTM D2500)	-21	0	

[a] D2: US low-sulfur No. 2 petroleum diesel fuel; Biodiesel: fatty-acid methyl-esters of soy oil.

The USBM heavy-duty transient test-cycle had an average load factor of approximately 40 %, with a number of fast accelerations at high engine load (> 50 % of peak torque) conditions. The conditions of this heavy-duty transient test-cycle often result in relatively high NO_x and high emissions of carbon-soot PM. In cases utilizing a DOC, the high temperatures of this transient test-cycle promote high oxidation of SO_2 to sulfate-PM (chiefly condensed sulfuric acid) and oxidation of NO to more toxic NO_2. For comparison purposes, the standard U.S. E.P.A. on-highway transient test-cycle [24] has an average load factor of approximately 20 %.

Transient test-cycle operation was chosen to approximate emissions generated during for loaded and unloaded haulage operations by mobile, diesel powered

mining production equipment. The transient cycles also allowed integration of certain emissions characteristics, such as PAH emissions, n-PAH emissions, and mutagenicity over a broad range of expected speed and load conditions.

The gaseous emissions measurement procedures used in this study are described in detail in McDonald *et al.* and Purcell *et al.* [16, 17]. In additions to the measurement of the usual range of gaseous emissions according to standard U.S. E.P.A. and ISO 8178 test protocols [22, 25], a number of other emissions were measured using a Fourier transform infrared (FTIR) exhaust spectrometer, including separate, direct measurement of NO, NO_2, NO_3, N_2O, and a number of organic species, including formaldehyde, acetaldehyde, and acrolein.

Sampling and analytical methods for characterization of PM emissions, and vapor-phase organic emissions are described in detail in McDonald *et al.*, Purcell *et al.* and Bagley *et al.* [16, 17, 18]. A Sierra BG-1 proportional, micro-dilution tunnel and PM sampling system was used for collection of PM samples using 90 mm diameter, Teflon-coated, fiberglass Pallflex filters during operation of the engines over the ISO 8178-C1 test-cycles [16]. Typical dilution ratios ranged from approximately 8:1 to approximately 16:1, depending on the exhaust temperatures and PM concentrations in the exhaust. The contribution of organic constituents to total PM was determined using purged vacuum oven sublimation, (PVOS) with a correction to account for volatilized sulfate material by separate determination of sulfate-PM using ion-chromatography.

During transient testing of the Caterpillar engine, an in-house, mini-dilution tunnel was used. PM samples were collected on 50 cm square Pallflex filters. A resin bed of XAD-2 polymer adsorbent was mounted immediately downstream of the PM-filter for collection of vapor-phase organic material. The contribution of organic constituents to total PM emissions during the transient testing was determined via soxhlet extraction with dichloromethane. For comparison purposes, organic PM emissions were also determined via PVOS with sulfate correction. For purposes of clarification, the solvent-extractable organic PM will always be referred to in this paper as PM-SOF, while the volatile organic PM determined by PVOS will be referred to as PM-VOC. Both methods give quite similar results and in the past have demonstrated similar trends in organic-PM emissions for a variety of test conditions [26].

Particle-size distributions for the PM emissions from the Caterpillar engine without the DOC were determined for both D2 and Biodiesel fuels over the steady-state speed and load conditions of the ISO 8178-C1 test-cycle. An electrical aerosol analyzer (EAA) was used to determine the particle-size distribution based on the electrical mobility distribution of the PM [27, 28, 29]. The mini-dilution tunnel was used with an ejector-diluter for secondary dilution. The final dilution ratios ranged from 280 to 360:1. The ejector-diluter was mounted approximately 6 meters (~0.5 seconds) downstream from the exhaust into the mini-dilution tunnel. The particle number concentrations versus particle-size were determined from EAA current distributions using the simplex minimization method developed by Kapadia [30]. Particle volume concentrations and the log-normal particle-size volume distributions were calculated from the particle number concentrations based on the methods of Hatch and Choate [31].

In the case of extracted organic material collected using the Caterpillar engine without the DOC, the alkane equivalent carbon number distribution of the vapor-phase organic material and the PM-SOF was determined using GC-FID methods. The extracted organic material was also analyzed for the presence of 9 polynuclear aromatic hydrocarbon (PAH) and 5 nitro-PAH compounds using HPLC with fluorescence detection [32, 33, 34]. The compounds included: fluoranthene (FLU), pyrene (PYR), benz[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), and benzo[a]pyrene (BaP), dibenz[a,h]anthracene (DBA), benzo[g,h,i]perylene (BP), 1-nitropyrene (1-NP), 2-nitrofluorene (2-NF), 6-nitrochrysene (6-NC), and 1,6-dinitropyrene (1,6-DNP) and 1,8-dinitropyrene (1,8-DNP). The compounds were chosen because of their suspected health effects, or because of their potential for conversion to compounds with suspected or know health effects [35].

Mutagenic activity of the PM and of the extractable vapor-phase organic compounds was determined using a modified version of the Ames mutagenicity bioassay, described in detail in Bagley *et al.* [18, 33]. All of the samples were tested using tester strain[4] TA98-S9, and, in some cases, with tester strains TA98NR-S9 and TA98-1,8-DNP_6 [33].

Results for PM Emissions and Extractable Organic Emissions

The PM-emissions trends were generally similar between the two transient test-cycles and the steady-state test-cycles for the tested conditions of fuel and exhaust aftertreatment. Figure 2 shows the brake-specific rates of PM emissions[5] and the contribution of PM-VOC for the fuels tested with the Caterpillar engine with no DOC over the ISO 8178-C1 test-cycle. The error bars represent a 95 % confidence interval (two-sided Student's t-test) for the weighted average PM emissions over the test-cycle. The Biodiesel fuel reduced PM emissions by approximately 31 % on average over the test-cycle, with a large decrease in solid, nonvolatile PM, and an increase in PM-VOC. Although the difference in total PM emissions for the B30 in comparison with the D2 fuel was not statistically significant at a confidence interval of 95 %, the B30 fuel exhibited a similar trend of shifting mass from solid PM to PM-VOC to that observed with the Biodiesel fuel.

The MWM engine produced somewhat higher PM emissions (figure 3), but with significantly lower PM-VOC in comparison with the Caterpillar engine. The Biodiesel fuel exhibited the same large decrease in solid, nonvolatile PM emissions. Although the increase in PM-VOC emissions with Biodiesel fuel using the MWM engine was proportionally similar to that of the Caterpillar engine, the overall lower levels of PM-VOC for the MWM engine resulted in much larger reductions in PM emissions for the Biodiesel fuel relative to the D2 fuel (48 %).

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[4] Specific *Salmonella typhimurium*/microsome mutagenicity test strain of bacteria.

[5] Brake-specific emissions are the mass-rate of emissions over an engine operating cycle, normalized by integrated work over the cycle.

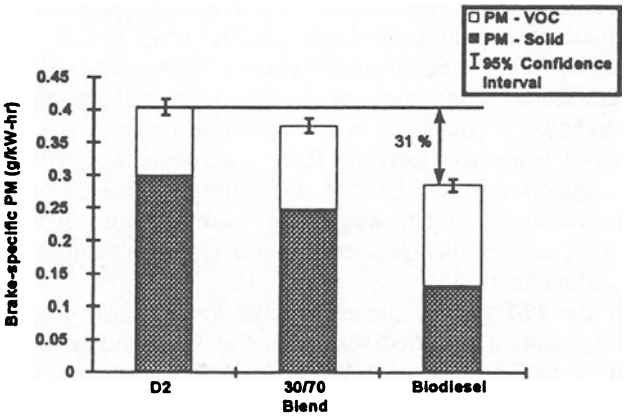

Figure 2. Brake-specific (work normalized) PM emissions for the Caterpillar 3304 PCNA, with no DOC, over the ISO 8178-C1 test cycle. The stacked bars show the contributions of VOC and solid material to PM. Error bars are for PM emissions.

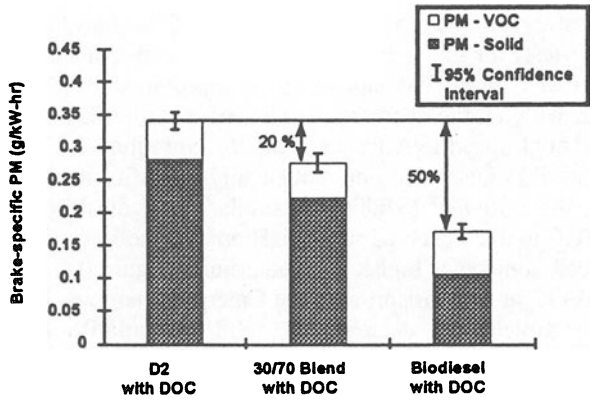

Figure 3. Brake-specific PM emissions for the Caterpillar 3304 PCNA, with DOC, over the ISO 8178-C1 test cycle.

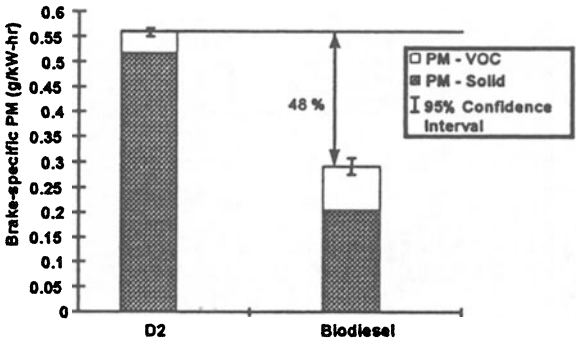

Figure 4. Brake-specific PM emissions for the MWM D-916-6 engine, with no DOC, over the ISO 8178-C1 test cycle.

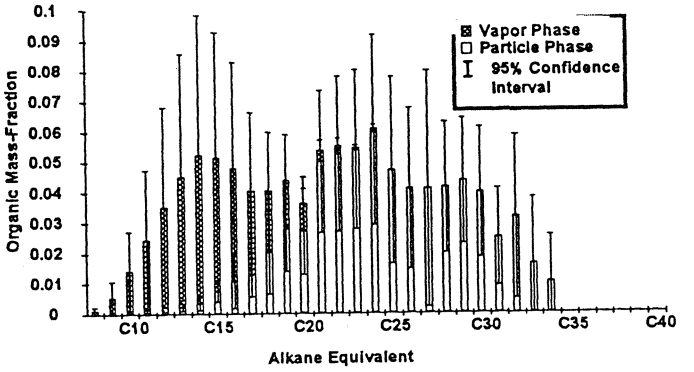

Figure 5. GC-FID alkane-equivalent carbon number distribution for the Caterpillar 3304 PCNA (no DOC) operating on D2 fuel over the USBM heavy-duty transient test-cycle. The distribution is expressed as a mass fraction of the total organic material collected and extracted (both particle and vapor-phase).

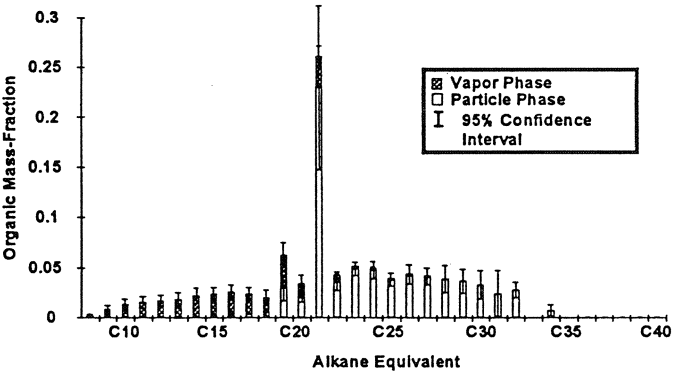

Figure 6. GC-FID alkane-equivalent carbon number distribution for the Caterpillar 3304 PCNA (no DOC) operating on Biodiesel fuel over the USBM heavy-duty transient test-cycle. Note the pronounced peaks at C19 and C21 alkane-equivalent, which are primarily in the particle-phase.

Previous experience with both engines suggests that somewhat lower oil consumption for the MWM engine relative to that of the Caterpillar engine may be in part responsible for the significant differences in the composition of the PM emissions.

The DOC proved quite effective in reducing PM-VOC for each of the fuels with the Caterpillar engine, each case resulting in lower PM than without the use of a DOC (figure 4). Emissions of sulfate-PM accounted for less than 3 % of the total PM emissions in the case of the D2 fuel with the DOC, and were not statistically significant for other conditions of fuel and exhaust aftertreatment. The Biodiesel and B30 fuels were much more effective as PM controls when used with a DOC. The Biodiesel fuel used with a DOC reduced PM emissions by approximately 50 % compared to the use of the D2 fuel with a DOC. The B30 fuel with a DOC reduced PM emissions by approximately 20 % compared to the D2 fuel with a DOC, and by approximately 35 % compared to D2 without the DOC.

The reasons for the shift towards higher PM-VOC or PM-SOF with Biodiesel fuels are readily apparent in the GC-FID chromatograms (figures 5 and 6). Although the uncertainty at a 95 % confidence interval was fairly high in the case of the chromatograms during use of the D2 fuel, certain general characteristics were identifiable and were consistent with previous investigations of this type using this engine [26]. While using the D2 fuel, the alkane-equivalent carbon number distribution was approximately bimodal, with the lower range of carbon numbers (approximately C8 to C18) representing partially burned or unburned fuel components, and with the higher range of carbon numbers (approximately C16 to C34) representing partially burned or unburned lubrication oil [26]. The

fuel-related organic compounds were found to be present primarily in the vapor- or gas-phase material extracted from the XAD-2 resin. The lubricating oil-related compounds were present primarily in the particle-phase extractable material. The high lubricating oil consumption of this engine was readily apparent in the chromatograms, and may be due to the design of the piston ring-pack and other oil consumption controls for the Caterpillar engine. It should be noted that this particular engine design predates most exhaust emission control regulations, and that engines for mining applications are specifically excluded from EPA off-highway exhaust emissions limits in the U.S. [22].

The chromatograms of the organic emissions were markedly different for the instances with Biodiesel fuel (figure 6). The fuel related organic compounds were present primarily within two peaks in the alkane-equivalent carbon-number distribution: C19 and C21. Compounds represented by these two carbon number peaks comprised more than 30 % of the organic compounds contributing to the PM-SOF. Further analysis of the soy-methyl-ester Biodiesel fuel via GC-FID yielded virtually identical peaks at C19 and C21 alkane equivalent carbon number. By comparison of GC-MS and HPLC results for the fuel to the GC-FID results for the fuel and the organic exhaust extractions, it was determined that the C19 peak was probably the result of methyl stearate and methyl palmitate exhaust emissions. The C21 peak was probably due to methyl oleate, methyl linoleate, and methyl linolenate exhaust emissions. It is important to note the carbon numbers expressed in the chromatograms are based on an alkane standard, and thus do not actually represent „true" carbon numbers, particularly for the partially unsaturated fatty-acid mono-ester compounds present in Biodiesel fuels.

When sampled from raw, hot exhaust, the organic emissions represented by FID-hydrocarbons (FID-HC) were somewhat lower overall for the Biodiesel fuel with either engine and with the B30 fuel in comparison to the D2 fuel. A typical example, the FID-HC emissions for the Caterpillar engine over the ISO 8178 test-cycle, is given in figure 7. Thus the increase in PM-SOF truly represented a shift in organic emissions from gas to particle-phase, not decreased combustion efficiency. Brake-specific fuel consumption was increased for the Biodiesel (14 to 15 %) and B30 fuels (4 %) due to their somewhat lower energy content, but cycle-average thermal efficiency[6] was nearly identical between the fuels tested with the Caterpillar or MWM engines.

Mutagenic activity of both the PM and the extracted vapor-phase organic material was significantly reduced by the Biodiesel fuel relative to the D2 fuel. The trends were not specific to engine load factor. An example of mutagenic activity corrected for engine power-output over the USBM light-duty transient cycle is given in figure 8.

Of the PAH and nitro-PAH compounds analyzed, seven PAH and one nitro-PAH compounds were present at levels above the minimum detection limits in the PM-SOF from the Caterpillar engine (figure 9). Three PAH compounds were present above the minimum detection limits in the extracted vapor-phase organic material (figure 10).

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[6] Also know as fuel conversion efficiency. Defined as $1/(BSFC*Q_{\text{Lower Heating Value}})$. It is an estimate of the thermodynamic engine cycle efficiency.

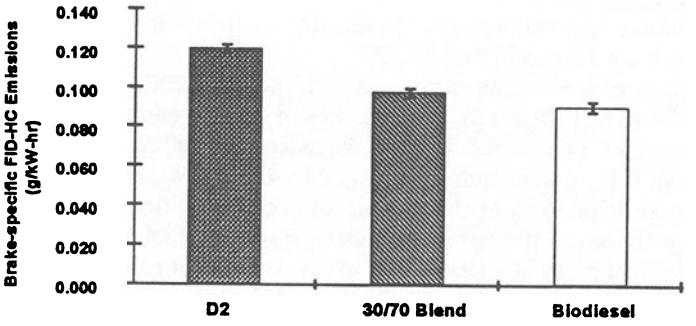

Figure 7. Brake-specific emissions of FID-hydrocarbons for the Caterpillar 3304 PCNA (no DOC) engine over the ISO 8178-C1 test cycle for each of the fuels tested.

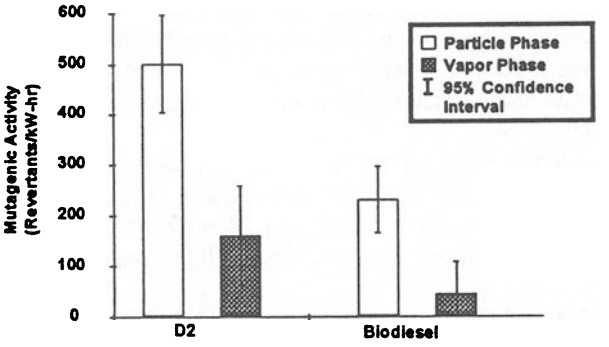

Figure 8. Mutagenic activity expressed as revertants corrected for the average engine power over the test cycle. Both PM and vapor-phase organic material are shown for the D2 and Biodiesel fuels. Caterpillar 3304 PCNA, USBM light-duty transient test cycle.

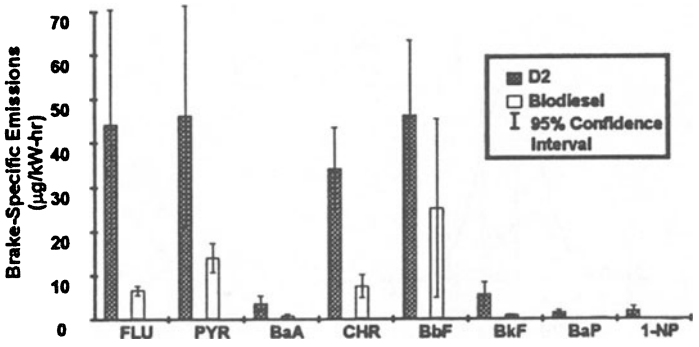

Figure 9. Brake-specific, particle-phase PAH and nitro-PAH emissions for both fuels. Caterpillar 3304 PCNA, USBM heavy-duty transient test-cycle.

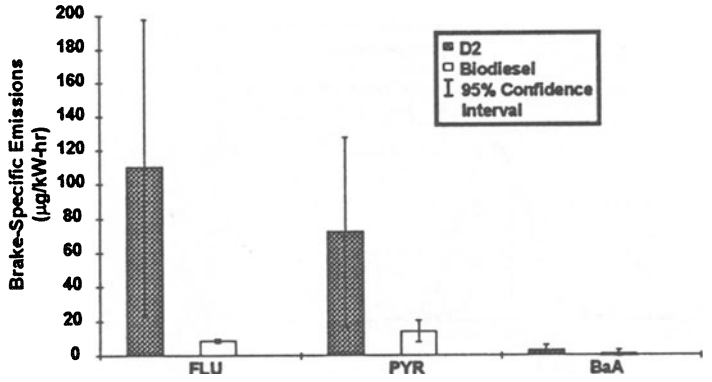

Figure 10. Brake-specific, vapor-phase PAH emissions for both fuels. Caterpillar 3304 PCNA, USBM heavy-duty transient test-cycle.

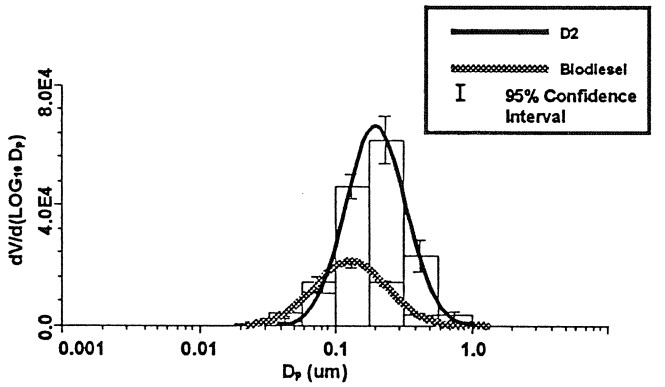

Figure 11. Particle volume concentration versus particle-size, Caterpillar 3304 PCNA, 100 % load, 1500 rpm.

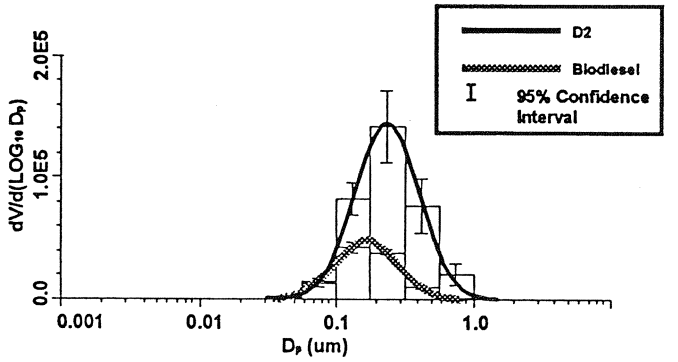

Figure 12. Particle volume concentration versus particle-size, Caterpillar 3304 PCNA, 100 % load, 2200 rpm.

There was a general trend of reductions in both particle- and vapor-phase brake-specific PAH and nitro-PAH emissions rates for the Biodiesel fuel, although these reduction were not always statistically significant at a 95 % confidence level. It is possible that the reduced levels of PAH and nitro-PAH compounds were responsible for the reduced mutagenic activity. It is also notable that on a basis of brake-specific emissions, the PM-associated mutagenic activity and PAH/nitro-PAH emissions were reduced overall while the PM-SOF was, in some cases, nearly doubled.

Figures 11 and 12 show the log-normal distribution of particle volume-concentration versus particle-size for two representative engine speed and load conditions. Trends were similar between the fuels at other tested engine conditions. The integrated area under the volume distribution curve represents total particle volume concentration, which is proportional to particle mass. Nuclei-mode particles did not contribute significantly to the particle volume concentrations within the particle-size range of the EAA (~0.01 to 1.0 μm). The reduction in particle volume concentration for the Biodiesel fuel was proportional to the reduction in solid PM (~60 %). The lower partial pressures of the organic material at such high dilution ratios would tend to favor evaporation of these species over adsorption or condensation, thus their contribution to particle mass or volume would be much lower under these conditions. With the reduction in PM-concentration using Biodiesel fuel, there was also a shift of the particle volume size-distribution toward a smaller volume mean-diameter. This was expected, since the reduced formation of primary soot particles due to the oxygen content of the fuel reduced the number of particles available in the exhaust for collision and growth by coagulation. The coagulation rate for an aerosol is related to particle number-concentration (N) by:

$$dN/dt = -KN^2$$

where K is the average coagulation coefficient, approximately 10 to 18 X 10^3 cm^3/s for the observed particle-size distribution characteristics of diluted PM exhaust emissions samples [36].

Gaseous Emissions Results

The reduction in brake-specific NO_x emissions for the Biodiesel fuel compared to the D2 fuel using the Caterpillar engine was somewhat unexpected (figure 13). The difference in NO_x emissions with the B30 fuel relative to the D2 fuel was not statistically significant. The NO_x reduction for the Biodiesel fuel was somewhat larger at light to moderate load conditions. NO formation is highly temperature dependent. Peak rates of cylinder pressure rise and a rapid increase in combustion temperature occurs during the pre-mixed phase of combustion. High NO emissions are often associated with high degrees of premixed combustion. The naturally aspirated Caterpillar engine during operation on the D2 fuel had a long ignition delay at part-load conditions, and a fairly high rate of pre-mixed combustion. These characteristics are somewhat atypical of most diesel engines with IDI combustion systems.

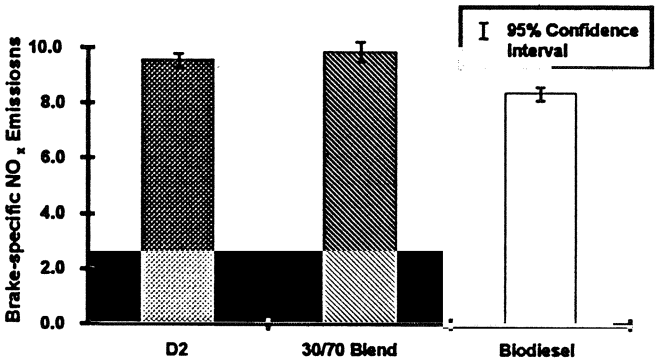

Figure 13. Brake-specific NO_x emissions over the ISO 8178-C1 test cycle for the Caterpillar 3304 PCNA.

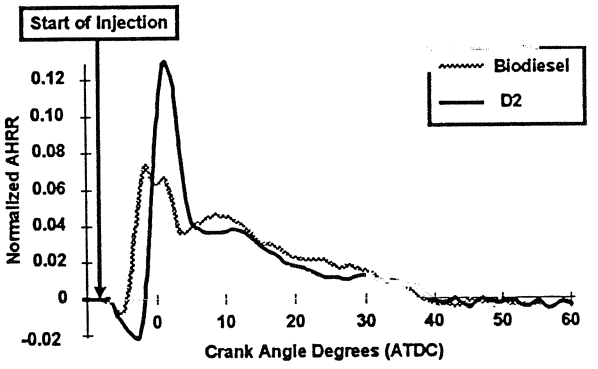

Figure 14. Normalized apparent rate of heat release. Caterpillar 3304 PCNA, 10 % load, 2200 rpm.

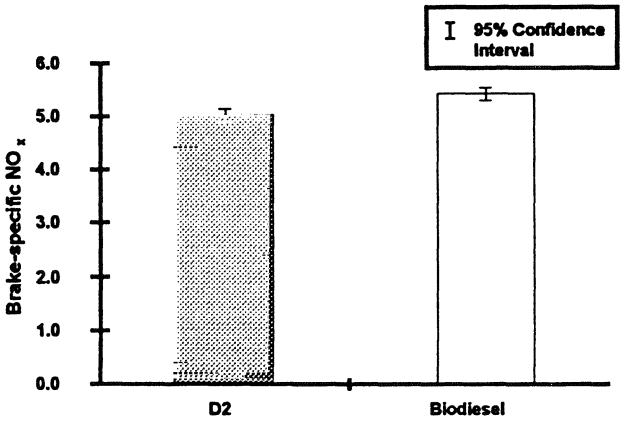

Figure 15. Brake-specific NO_x emissions over the ISO 8178-C1 test cycle for the MWM D916-6 engine.

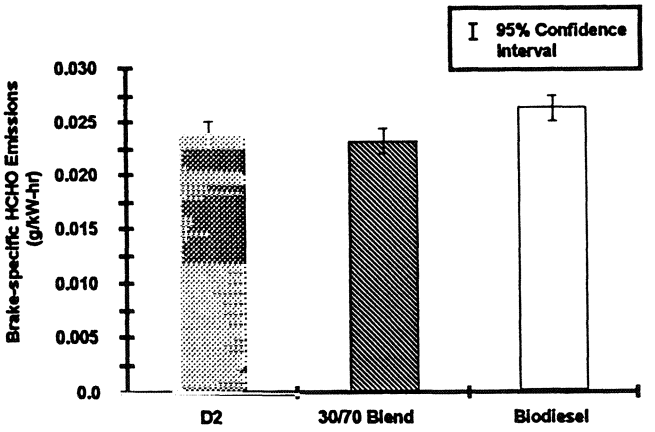

Figure 16. Brake-specific formaldehyde (HCHO) emissions over the ISO 8178-C1 test cycle for the Caterpillar 3304 PCNA.

The higher cetane number and shorter ignition delay of the Biodiesel resulted in a significant reduction in the premixed-burn fraction of combustion, the maximum rate of heat release during premixed combustion, and the calculated combustion temperatures during high speed, light and part load conditions. An example of an extreme case, 10 % load, 2200 rpm (figure 14), illustrates the difference in ignition delay.

The NO_x emissions from the MWM engine were slightly increased (3.1 %) for the Biodiesel fuel over the D2, but the difference was still statistically significant (figure 15). The combustion system of the MWM uses a somewhat more advanced swirl-chamber design (modified Comet-type swirl-chamber) compared to the relatively coarse pre-chamber used by the Caterpillar. It is possible that the MWM combustion system exhibited the small or nearly absent premixed burn fraction and short ignition delay typical of more modern IDI combustion systems [37] rather than the atypical characteristics exhibited with the Caterpillar engine. This may be partially responsible for different NO_x emissions results between the two engines, but the results are currently inconclusive due to the lack of comparable combustion data for the MWM engine.

Formaldehyde emissions (figure 16) were slightly increased by the Biodiesel fuel relative to the D2 or B30 fuels on a brake-specific basis, but exhaust concentrations of formaldehyde were extremely low for all of the fuels over the test test-cycles utilized. Concentrations of acrolein and acetaldehyde in the exhaust were too low for accurate determination via FTIR.

Conclusions

A detailed characterization of exhaust emissions from two Biodiesel fueled engines, including particle-size analysis, determination of exhaust mutagenic activity, identification of PAH, nitro-PAH, and some aldehyde compounds, did not demonstrate any significant detriment to the use of Biodiesel fuel. The reductions in carbon-soot emissions, and the reductions in total PM emissions when Biodiesel fuels are used with a DOC, suggest that the fuel could be used as an effective passive PM control for diesel engines used in confined areas such as underground mines.

The variations in NO_X emissions between various engine applications are still not fully understood. In turbocharged diesel engines with modern high-pressure, direct-injection (DI) combustion systems, there appears to be a clear relationship between oxygen availability during combustion, including fuel oxygen from oxygenated diesel fuels such as Biodiesel, and significantly higher NO formation due to increased flame temperatures [8, 9, 12, 38, 39]. This does not appear to be the case with some IDI engine applications, and the effects of Biodiesel fuel on ignition delay appeared to play a significant role in reducing the NO_x emissions from the Caterpillar engine tested as part of this program.

The relationship between the formation of PM-SOF and carbon number suggests that some modification of the plant oil towards lower carbon number fatty-acids might reduce PM-SOF formation. Although this appears to be

technically feasible, the PM-SOF emissions were controllable through the use of a DOC. DOCs are now in widespread use in light-duty diesel applications and in some heavy-duty applications such as underground mines.

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References

1. Mine Safety and Health Administration, U.S. Department of Labor, U.S. Federal Register **57**, 3 500-503 (1992).
2. American Conference of Governmental Industrial Hygienists, *ACGIH Today* **3**, 1 (1995).
3. W.F. Watts, Jr., *Assessment of Occupational Exposure to Diesel Emissions* in *Diesel Exhaust: A Critical Analysis of Emissions, Exposure, and Health Effects*, Health Effects Institute, Cambridge MA, USA 106-123 (1995)
4. R.A. Haney, *Society of Mining Engineers Pre-Print* **90-40** (1990).
5. M.K. Gangal and E.D. Dainty, *Proceedings of the 6th U.S. Mine Ventilation Symposium* (R. Bhaskar, ed.) Society for Mining, Metallurgy and Exploration, 83-89 (1993).
6. W.F.Watts, Jr., B.K. Cantrell, J.L. Ambs, K.L. Rubow, *U.S. Bureau of Mines I.C.* **9324**, 31-39 (1992).
7. O.G. Raabe, *Ann. American Conference of Industrial Hygienists* **11**, 55 (1984).
8. F.J. Liotta and D.M. Montalvo, *SAE Tech. Pap.* **932734** (1993).
9. T.L. Ullman, K.B. Spreen, R.L. Mason, *SAE Tech. Pap.* **941020** (1994).
10. J.E. Dec, A.O. zur Loye, D.L. Siebers, *SAE Tech. Pap.* **910224** (1991).

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[7] Contract number USDI-1432 C0369004.
[8] Contract number BOM-CRDA-6200-0063
[9] Contract number BOM-MOA-14-09-0070-1587
[10] Contract number 93-COOP-1-9554

11. J.B. Heywood, *Internal Combustion Engine Fundamentals* (McGraw-Hill Publ. Co.), pp. 633-642 (1988).
12. N. Ladommatos, S.M. Abdelhalim, H. Zhao, Z. Hu, *SAE Tech. Pap.* **961165** (1996).
13. J.R. Needham and D.M. Doyle, *SAE Tech. Pap.* **852101** (1985).
14. R.J. Last, M. Kruger, M. Durnholz, *SAE Tech. Pap.* **950054** (1995).
15. K. Schmidt and J. Van Gerpen, *SAE Tech. Pap.* **961086** (1996).
16. J.F. McDonald, D.L. Purcell, B.T. McClure, D.B. Kittelson, *SAE Tech. Pap.* **950400** (1995).
17. D.L. Purcell, B.T. McClure, J. McDonald, H.N. Basu, *J. Am. Oil Chem. Soc.* **75**, 381 (1996)
18. S.T. Bagley, L.D. Gratz, J.H. Johnson, J.F. McDonald, *Env. Sci. and Tech.* (publication pending - 1997).
19. R.B. Krieger and G.L. Borman, *ASME* **66-WA/DGP-4** (1966).
20. C.F. Anderson, J.D. Gage, M.J. Vogel, N.D. Lange, *U.S. Bureau of Mines I.C.* **9324** 91-102 (1992).
21. International Organization for Standardization, International Standard ISO/DIS 8178-4 (1996)
22. U.S. Code of Federal Regulations, 40 CFR 89 (1996).
23. B.T. McClure, S.T. Bagley, L.D. Gratz, *SAE Tech. Pap.* **920854** (1992).
24. U.S. Code of Federal Regulations, 40 CFR 86 - Subpart N (1996).
25. International Organization for Standardization, International Standard ISO/DIS 8178-1 (1996)
26. J.E. Johnson, *Hydrocarbon Oxidation in a Diesel Catalytic Converter* University of Minnesota M.S. Thesis (1993).
27. D.B. Kittelson, D.F. Dolan, R.B. Diver, E. Aufderheide, *SAE Tech. Pap.* **780787** (1978).
28. K.J. Baumgard and D.B. Kittelson *SAE Tech. Pap.* **850009** (1985).
29. D.B. Kittelson, J. Reinertsen, J. Michalski, *SAE Tech. Pap.* **910329** (1991).
30. A. Kapadia, *Data Reduction Techniques for Aerosol Size Distribution Measuring Instruments*, University of Minnesota Particle Tech. Lab. Pub., **413**, June (1980).
31. T. Hatch and S.P. Choate, *J. Franklin Inst.* **207** (1929).
32. L.D. Gratz, S.T. Bagley, K.S. King, K.J. Baumgard, D.G. Leddy, J.H. Johnson, *SAE Tech. Pap.* **910609** (1991).
33. S.T. Bagley, K.J. Baumgard, L.D. Gratz, J.H. Johnson, D.G. Leddy, *Health Effects Institute* **76** (1996).
34. W.A. MacCrehan, W.E.May, S.D. Yung, B.A. Benner, Jr., *Anal. Chem* **60** 194-199 (1988).
35. World Health Organization, International Agency for Research on Cancer, *IARC Monographs* **46** (1989).
36. K.W. Lee and H. Chen, *Aerosol Sci. Tech.* **3**, 327-334 (1984).
37. J.B. Heywood, *Internal Combustion Engine Fundamentals* McGraw-Hill Publ. 503-517 (1988).
38. N. Ladommatos, S.M. Abdelhalim, H. Zhao, Z. Hu, *SAE Tech. Pap.* **961167** (1996).
39. R.R. Sekar and W.W. Murr, *SAE Tech. Pap.* **901565** (1990).

Toxicology and Ecotoxicology of Biodiesel Fuel

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Abstract

This work attempts to demonstrate the toxic and ecotoxic hazards of Biodiesel and diesel petroleum fuel. The hazard was estimated by means of unispecies-testing, by testing a microcosm and by analysing the biodegradability. In the first test Biodiesel and diesel were applied directly to animals or to the medium the animals or plants lived in, during the second test Biodiesel and diesel werde eluated and the residue was tested for toxic effects. The results of the effects of these two oil types referring to mammals, fishes, evertebrates, higher plants, algae, cyanobacteria, microbiological populations and bacteria were shown and the toxicity and ecotoxicity have been assessed for each taxonomic group as well. Substantiated by the toxicity/ecotoxicity/biodegradability-data results showed, that Biodiesel is much better to environment when compared to petroleum diesel fuel.

Introduction

In the year 1990, the Federal Institute for Water Quality was invited by the Federal Institute of Agricultural Engeneering, Wieselburg, Austria, to make a contribution referring to the behaviour of Biodiesel in the environment. There was no literature to be found in the field of toxicity of Biodiesel and as well as petroleum diesel fuel. Therefore we investigated and decided to compare the results derived from toxicologic analysis of Biodiesel with data of similar investigations concerning petroleum diesel fuel

At this time it was discussed wether Biodiesel was proper for vehicles in traffic and wether there is any advantage in using Biodiesel as compared to petroleum diesel fuel. This question also included matters referring the hazard of using this new material.

Meanwhile there is no doubt in using Biodiesel in public and private cars and special machines. Some questions about the effects of Biodiesel on men and the environment and how we formulate new questions in context at improving the technical performance and chronic effects of Biodiesel in ecology.

This paper it is an attempt to present the recent data of toxicity and ecotoxicity of Biodiesel, in as far as they were available.

Material and methods

Fatty acid methyl esters, saturated or unsaturated with even numbers and no ramification of the C-chain and C-numbers greater than 6 were defined to be one group in order to interprete their potential risk to water. In this way it seems to be possible to compare Biodiesel derived from rape methylester or Soydiesel or any other Biodiesel on the one side with petroleum diesel fuel.

In order to assess the toxic effects of Biodiesel it was directly applied to mammals or man. It was more difficult to apply this material to aquatic organisms when analyzing the ecotoxicity. Some investigators added Biodiesel after a certain adaptation period (24, 48 hours), others ran biotests with eluated Biodiesel (24 hours).

The organisms within the unispecies tests were:

Consumers	Mammals	Man, rats, rabbits
	Fishes	Rainbow trout, bluegills
	Invertebrates	Crustacea: *Daphnia magna, Daphnia pulex, Gammarus pulex*
		Molluscs: *Lymnea peregra*
Producers	Higher plants	*Lemna minor, Lemna minuta, Elodea canadensis, Myriophyllum spicatum*
	Algae	*Selenastrum capricornutum, Chlorella vulgaris, Nannochloris coccoides*
	Cyanobacteria	*Microcystis aeruginosa, Anabaena spiroides*
Destruents	Bacteria	*Pseudomonas putida*
	Mixed microbiological populations	*Bacteria, Fungi, Protozoa*

The microcosm was fitted together with:

Consumers	Fish	*Gasterosteus aculeatus, Pimephales promelas, Oncorhynchus mykiss*
	Invertebrates	*Daphnia magna, Lymnea peregra*
	Higher plants (*Spermatophyta*)	*Elodea canadensis, Myriophyllum spicatum*

Most of the investigations followed standard methods but also own methods to assess the toxicity were used.

- The Austrian Research Centre, Seibersdorf (ÖFZS), investigated the dermal toxicity by applying the OECD Guidline 402, „Acute dermal toxicity, Limit-test". The oral toxicity was proved by applying the OECD Guidline 401, „Acute oral toxicity, Limit-test".
- The Experimental Institute for Biochemistry and Controlling of injurious organisms (HBLV-Ch) made its biodegradation analyses by CEC L-33-T-82
- The Institute of Arable Crops Research - Long Ashton Research Station did not give any national or international references referring to its applied methods.
- Information about toxicity and biodegradabilty were provided by Midwest Biofuels Inc in form of a Biological Safety Summary without any expression of methods used.
- The Federal Institute for Water Quality (IWG) ran its tests with fish, crustacea, green algae, higher plants, bacteria, microbiological populations by using ÖNORM M 6263 „Bestimmung der akuten Toxizität von Wasserinhaltsstoffen gegenüber *Salmo gairdneri* Richardson (Regenbogenforelle). Semistatischer Test" 1987,
  ÖNORM-EN 26341 „Wasserbeschaffenheit. Bestimmung der Bewegungshemmung von *Daphnia magna* Straus (Cladocera, Crustacea). Bestimmung der akuten Toxizität" 1994,
  ISO 8692 „Water quality - Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*" 1989,
  Method by Neururer „Abgeänderter Kressewurzeltest" 1975,
  ISO/DIS 10712.2 „Water quality - *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test) 1995 and
  ÖNORM B 5104 „Abwasserverhalten von Kaltreinigungsmitteln („Kaltreinigern") für KFZ- und Motorenreinigung - Pkt. 8.4.2. Bakterientest" 1988.

Results

Tabels 1.1 through 2.2.6 will show data resulting from toxicity and ecotoxicity assessments with Biodiesel/Biodiesel eluate and also petroleum diesel fuel/petroleum diesel fuel eluate. The tabels are divided in fields of:

- Toxicity assessment: i.e. reactions of mammals referring to their skin, eye, inhalation-organs and digestion-organs (oral);
- Ecotoxicity assessment i.e. reactions of producers (different algae and higher plants), consumers (fish, crustaceans and gastropods), destruents (bacteria and microbiological populations) and of a microcosm, and also;
- Biodegradability

The first column of these tables lists the test criteria. In the second column, called „Investigator" one can find the short form of the name of the laboratory that performed the analyses. These were:

Höhere Bundes-Lehr- und Versuchsanstalt für Chemische Industrie	HBLV-Ch
Long Ashton Research Station, Institute of Arable Crops Research	ASHTON
Midwest Biofuels INC	MBI
Institut für Wassergüte des Bundesamtes für Wasserwirtschaft	IWG
Österreichisches Forschungszentrum Seibersdorf	ÖFZS
OMV - AG.	OMV

The third column shows how the oil was applicated to the test organisms. In the fourth column the full name of the organisms used in the investigations is listed. Last the fifth column reports the results and is presented in concentrations of grams per litre. In order to get a good base for comparisons, the ED/EC10 and also the ED/EC50 results werde listed, if available. Attention should be paid to the fact, that greater numbers (concentrations) stand for lower toxicity!

Table 1.1. Biodiesel; undiluted C16-C18 methylesters: *Toxicity tests - Skin, Eye, Oral*

Test	Investigator	Application	Test organism	LD/LC g/kg
Skin irritation/Acute dermal toxicity	MBI	Biodiesel applied directly	man	Material produces very mild irritattion
	ÖFZS	Biodiesel applied directly	rat	LD 50 = > 2
Eye irritation	MBI	Biodiesel applied directly	rabbit	LD - Undiluted material caused mild transient irritation. In one day or less the eye returned to normal.
Acute oral toxicity	MBI	Biodiesel applied directly	rat	LD 10 = > 17,4
	ÖFZS	Biodiesel applied directly	rat	LD 50 = > 2

Table 1.2.1. Biodiesel; undiluted C16-C18 methylesters: Ecotoxicity tests - Producers, Algae

Test	Investigator	Application	Test organism	ED/EC g/l
Cyanobacteria growth	ASHTON	Biodiesel added to cultures	*Microcystis aeruginosa*	EC10 = 0,15 EC50 = 1,03
Cyanobacteria growth rate	ASHTON	Biodiesel added to cultures	*Microcystis aeruginosa* *Anabaena spiroides*	EC10 = 0,7 EC50 = 7,0 EC10 = 17,6 EC50 = >>17,6
Algal growth	ASHTON	Biodiesel added to cultures	*Selenastrum capricornutum* *Chlorella vulgaris* *Nannochloris coccoides*	EC10 = 0,72 EC50 = 7,1 EC10 = 0,0006 EC50 = 11,6 EC10 = 0,19 EC50 = 3,1
Algal growth	IWG	Biodiesel-eluate	*Selenastrum capricornutum*	EC10 = > 174 EC50 = > 174
Algal growth rate	ASHTON	Biodiesel added to cultures	*Selenastrum capricornutum* *Chlorella vulgaris* *Nannochloris coccoides*	EC10 = 0,03 EC50 = 5,4 EC10 = 0,46 EC50 = 1162,0 EC10 = 0,76 EC50 = 26,7

Table 1.2.2. Biodiesel; undiluted C16-C18 methylesters: Ecotoxicity tests - Producers, Higher plants

Test	Investigator	Application	Test organism	EC g/l
Macrophyts growth in Petri dishes	ASHTON	Biodiesel added to cultures	*Lemna minor* (duckweed)	EC10 = 0,1 EC50 = 23,4
			Lemna minuta	EC10 = 0,06 EC50 = 29,5
Macrophyts growth in jars	ASHTON	Biodiesel added to cultures	*Lemna minor*	EC10 = - EC50 = 0,001
			Lemna minuta	EC10 = 0,001 EC50 = 16,4
Macrophyts growth - number of green fronds	ASHTON	Biodiesel added to cultures	*Lemna minor*	EC10 = 0,004 EC50 = 0,04
			Lemna minuta	EC10 = 0,15 EC50 = 1,5
Macrophyts growth	IWG	Biodiesel-eluate	*Lepidium sativum* (cress)	EC10 = >174 EC50 = >174
Macrophyts growth	ASHTON	Biodiesel added to water surface	*Elodea canadensis*	EC10 = - EC50 = - No distict dose effects
			Myriophyllum spicatum	EC10 = - EC50 = - No distict dose effects
Macrophyts growth	ASHTON	Biodiesel added to hydrosol	*Elodea canadensis*	EC10 = - EC50 = 0,05
			Myriophyllum spicatum	EC10 = - EC50 = - No distict dose effects

Table 1.2.3. Biodiesel; undiluted C16-C18 methylesters: Ecotoxicity tests - Consumers

Test	Investigator	Application	Test organism	ED/EC g/l
Fish mortality	IWG	Biodiesel eluate	*Oncorhynchus mykiss* (rainbowtrout)	48 hEC10 = >174 48 hEC50 = >174
	MBI	Soydiesel	*Lepomis macro-chirus* (Bluegill)	LC50 = - LC50 = > 1
Daphnia - Inhibition of mobility	ASHTON	Biodiesel/water mixture	*Daphnia magna* (water flea)	24hEC10 = 0,1 24hEC50 = 9,1 48hEC10 = 0,1 48hEC50 = 23,3
			Gammarus pulex	24hEC10 = 0,8 24hEC50 = 8,1 48hEC10 = 0,5 48hEC50 = 2,0 72hEC10 = - 72hEC50 = 1 96hEC10 = - 96hEC50 = 1
			Lymnea peregra	24hEC10 = > 100 24hEC50 = >>100 48hEC10 = < 100 48hEC50 = > 100 72hEC10 = << 50 72hEC50 = 50 96hEC10 = 10,3 96hEC50 = 32,1
Daphnia - Inhibition of mobility	IWG	Biodiesel-eluate	*Daphnia magna*	24hEC10 = 122 24hEC50 = 136

Table 1.2.4. Biodiesel; undiluted C16-C18 methylesters: Ecotoxicity tests - Destruents

Test	Investigator	Application	Test organism	EC g/l
Bacterial growth	IWG	Biodiesel-eluate	*Pseudomonas putida*	16hEC10 = >435 16hEC50 = >435
Microbio-logical po-pulation - physiologi-cal effect	IWG	Biodiesel-eluate	Microbiological population	Furtherance of BOD up to more than 150 % at 174
	MBI	Soydiesel	Microbiological population	No inhibition up to 10

Table 1.2.5. Biodiesel; undiluted C16-C18 methylesters: Ecotoxicity tests - Microcosm

Test	Investigator	Application	Test organism	Effect
Fish - average loss of weight	ASHTON	Biodiesel added to water surface	Gasterosteus aculeatus Pimephales promelas	Effects only rarely different from the control group
Partitition into components		Biodiesel added to water surface	Physical and chemical circumstances	Biodiesel formed globules onto surfaces where they deposited as a white waxy residue, noticed visually after <7 days. This oil permeats very rapidly the whole micocosm
Persistance		Biodiesel added to water surface		The tracking ion for Biodiesel enters the microcosm from the surface globules more quickly than the diesel ion enters from the surface slick. The data show that the Biodiesel appears to disappear (degrade) more quickly than the marine diesel.

Table 1.2.6. Biodiesel (RME): Biodegradability tests

Test	Investigator	Application	Test organism	Biological degradability %
Microbiological population	HBLV-Ch	Biodiesel	Microorganims from a waste water plant	99,3
		Additiv for Biodiesel	Microorganims from a waste water plant	97,8
		Biodiesel with additives	Microorganims from a waste water plant	100

Table 2.1. Petroleum diesel fuel: Toxicity tests - Skin, Eye, Inhalation, Oral

Test	Investigator	Application	Test organism	LD/LC g/kg
Skin irrita-tion/Acute dermal toxicity	OMV	Diesel applied directly	man	Longer contact results dermatitits
Eye irritation	OMV	Diesel applied directly	man	Coniunctivitis
Inhalation	OMV	Diesel vapour	rat	LC50 = > 5g/m3
Acute oral toxicity	OMV	Diesel applied directly	man rat rabbit	TD = 0,084-0,168 LD50 = > 5 LD50 = > 2

Table 2.2.1. Petroleum diesel fuel: Ecotoxicity tests - Producers, Algae

Test	Investigator	Application	Test organism	EC g/l
Cyanobacteria growth	ASHTON	Diesel added to cultures	*Microcystis aeruginosa* *Anabaena spiroides*	EC10 = 0,049 EC50 = 0,55 EC10 = <100 EC50 = <100
Cyanobacteria growth rate	ASHTON	Diesel added to cultures	*Microcystis aeruginosa* *Anabaena spiroides*	EC10 = 0,343 EC50 = 6,34 EC10 = 0,68 EC50 = 4,8
Algal growth	ASHTON	Diesel added to cultures	*Selenastrum capricornutum* *Chlorella vulgaris* *Nannochloris coccoides*	EC10 = <1 EC50 = <1 EC10 = <20 EC50 = <20 EC10 = 0 EC50 = 0,004
Algal growth	IWG	Diesel-eluate	*Selenastrum capricornutum*	EC10 = 0,084 EC50 = 0,202
Algal growth rate	ASHTON	Diesel added to cultures	*Selenastrum capricornutum* *Chlorella vulgaris* *Nannochloris coccoides*	EC10 = <1 EC50 = <1 EC10 = 0,0001 EC50 = 349 EC10 = 0,003 EC50 = 3,74

Table 2.2.2. Petroleum diesel fuel: Ecotoxicity tests - Producers, Higher plants

Test	Investigator	Application	Test organism	EC g/l
Macrophyts growth in Petri dishes	ASHTON	Diesel added to cultures	*Lemna minor*	EC10 = 0,7 EC50 = 18,9
			Lemna minuta	EC10 = 0,27 EC50 = 2,18
Macrophyts growth in jars	ASHTON	Diesel added to cultures	*Lemna minor*	EC10 = <0,0125 EC50 = <0,0125
			Lemna minuta	EC10 = 0,015 EC50 = 0,25
Macrophyts growth - number of green fronds	ASHTON	Diesel added to cultures	*Lemna minor*	EC10 = <0,0125 EC50 = 0,0125
			Lemna minuta	EC10 = 0,0001 EC50 = 0,004
Macrophyts growth	IWG	Diesel-eluate	*Lepidium sativum* (cress)	EC10 = 42,0 EC50 = >168
Macrophyts growth	ASHTON	Diesel added to water surface	*Elodea canadensis*	EC10 = - EC50 = - No distict dose effects
			Myriophyllum spicatum	EC10 = <<0,0125 EC50 = <0,0125
Macrophyts growth	ASHTON	Diesel added to hydrosol	*Elodea cana-densis*	EC10 = 1,55-2,02 EC50 = 10,7-15,2
			Myriophyllum spicatum	EC10 = - EC50 = 0,06

Table 2.2.3. Petroleum diesel fuel: Ecotoxicity tests - Consumers

Test	Investigator	Application	Test organism	ED/EC g/l
Fish mortality	IWG	Diesel eluate	*Oncorhynchus mykiss* (rainbowtrout)	48hEC10 = 2,52 48hEC50 = 5,04
Daphnia - Inhibition of mobility	ASHTON	Biodiesel/water mixture	*Daphnia magna*	24hEC10 = << 1 24hEC50 = << 1 48hEC10 = << 1 48hEC50 = << 1
			Gammarus pulex	24hEC10 = << 1 24hEC50 = << 1 48hEC10 = << 1 48hEC50 = << 1 72hEC10 = << 1 72hEC50 = << 1 96hEC10 = << 1 96hEC50 = << 1
			Lymnea peregra	24hEC10 = < 1 24hEC50 = < 1 48hEC10 = < 1 48hEC50 = < 1 72hEC10 = << 1 72hEC50 = << 1 96hEC10 = << 1 96hEC50 = << 1
Daphnia - Inhibition of mobility	IWG	Diesel-eluate	*Daphnia magna*	24hEC10 = 10,08 24hEC50 = 47,04

Table 2.2.4. Petroleum diesel fuel: Ecotoxicity tests - Destruents

Test	Investigator	Application	Test organism	EC g/l
Bacterial growth	IWG	Diesel-eluate	*Pseudomonas putida*	16hEC10 = >2,18 16hEC50 = >168
Microbiological population - physiological effect	IWG	Diesel-eluate	Microbiological population	Inhibition of BOD at 168

Table 2.2.5. Petroleum diesel fuel: Ecotoxicity assessment - Microcosm

Test	Investigator	Application	Test organism	Effect
Fish - average loss of weight	ASHTON	Diesel added to water surface	*Gasterosteus aculeatus Pimephales promelas*	LOEC = 0,02
Partitition into components		Diesel added to water surface	Physical and chemical circumstances	The behaviour of this oil is different from this of the Biodiesel
Persistance		Diesel added to water surface		The Diesel appears to dis-appear (degrade) more slowly than Biodiesel

Table 2.2.6. Petroleum diesel fuel: Biodegradability tests

Test	Investigator	Application	Test organism	Biological de-gradability %
Microbiologi-cal population	HBLV-Ch	Diesel	Microorganims from a waste water plant	72,0

Diagrams one to three summarize relevant data for EC 10 and EC 50 of cyanobacteria, algae, macrophyta and consumers, destruents, micopopulations when investigating Biodiesel and diesel and the similar data when investigating the oil eluates.

Diagram four shows the averages from EC 10 data (Cyanobacteria, algae, macrophyts, destruents) and EC 50 data (consumers), which were relevant for the ecotoxicity assessment.

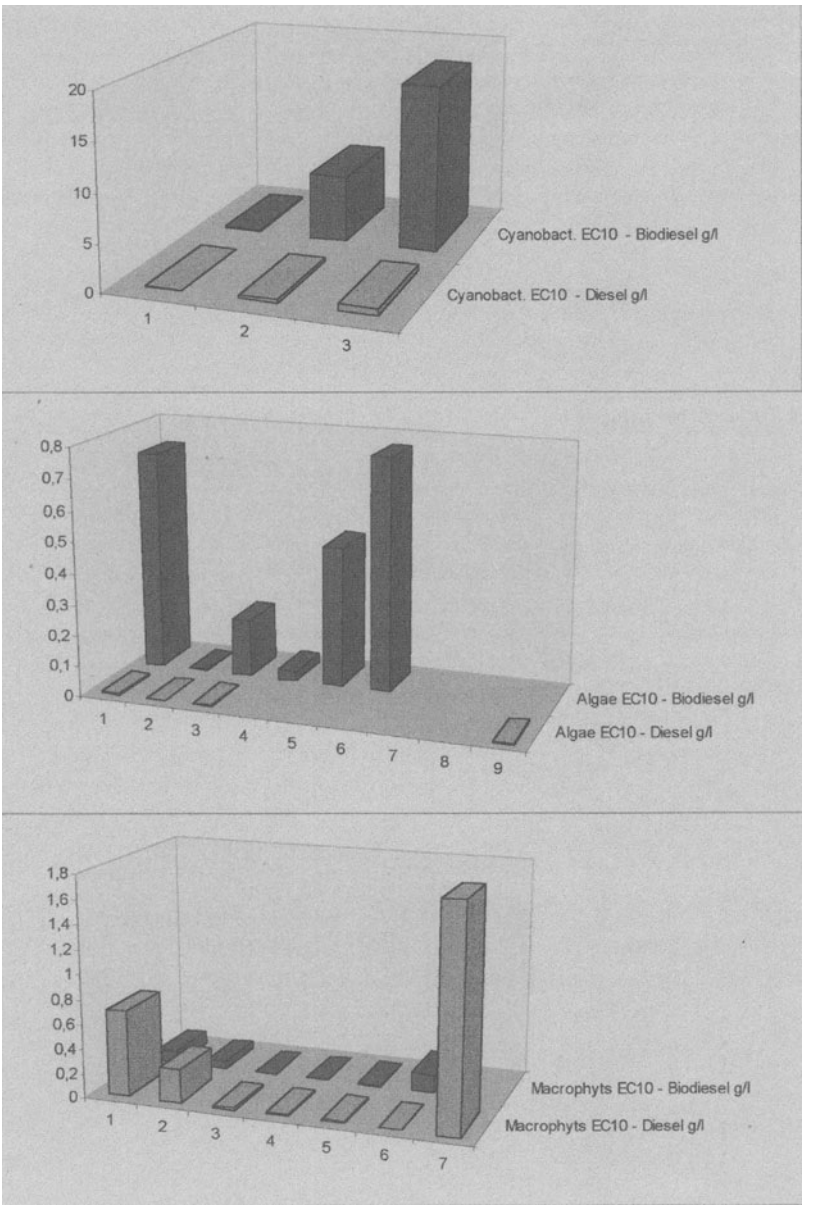

Diagram 1. Difference between Biodiesel and petroleum diesel fuel referring to relevant EC10 data of plantal producers (ordinate: g/l fuel; abscissa: different investigastions)

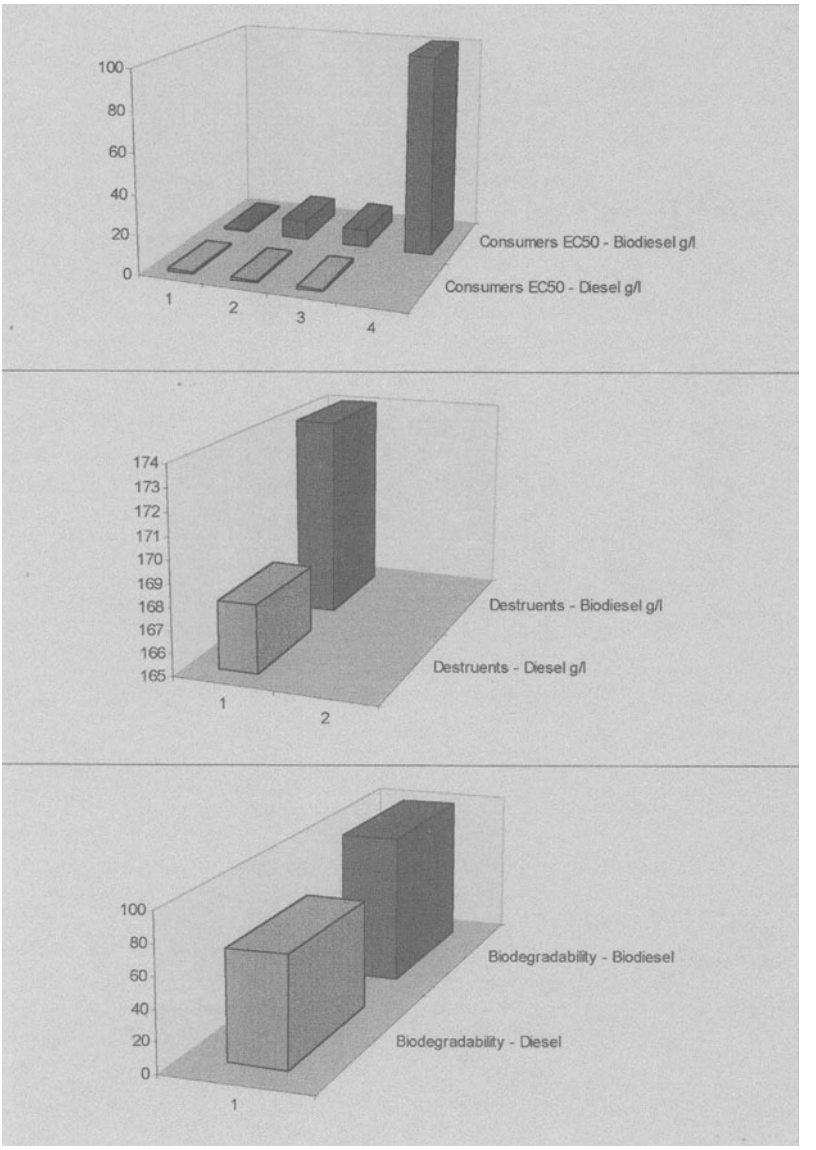

Diagram 2. Difference between Biodiesel and petroleum diesel fuel referring to relevant EC10 data (destruents), EC50 data (animal consumers) and data of Biodegradability (ordinate: g/l fuel; abscissa: different investigastions)

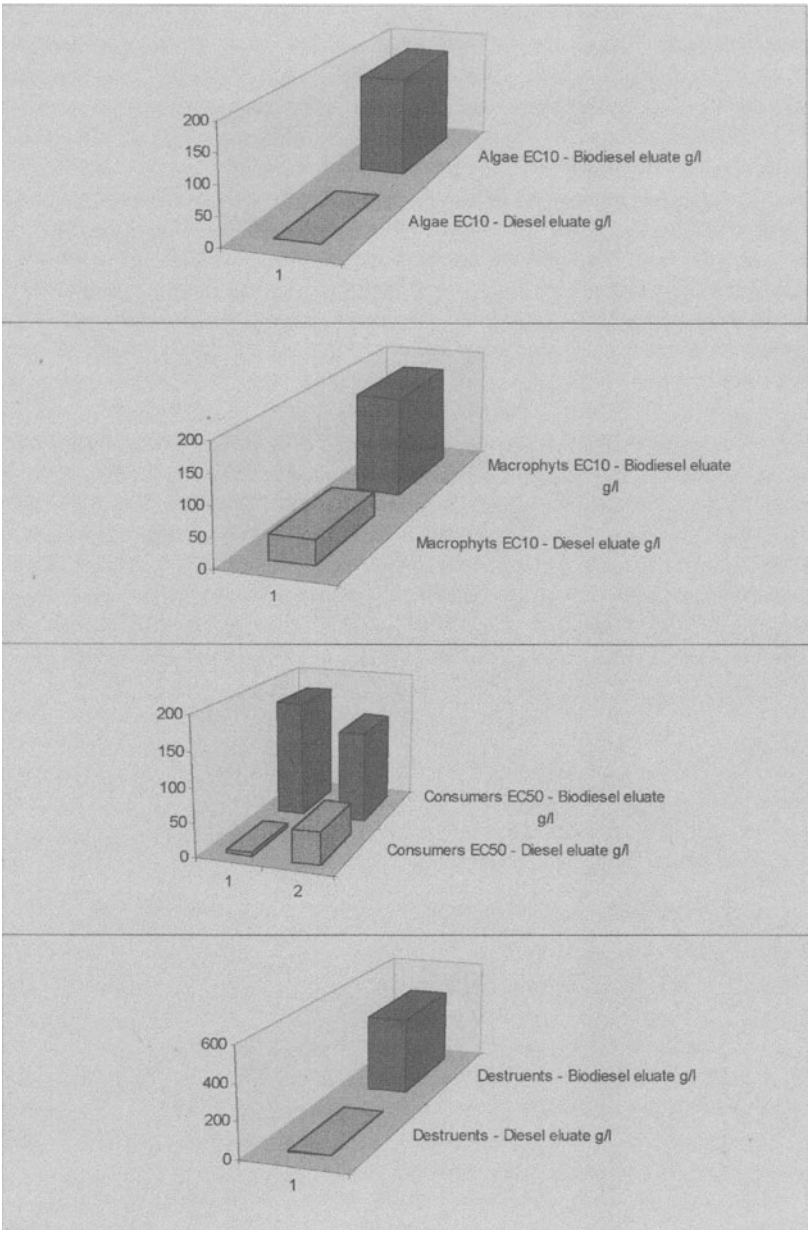

Diagram 3. Difference between Biodiesel eluate and petroleum diesel fuel-eluate referring to relevant EC10 data (algae, macrophyts, destruents) and EC50 data (consumers) (ordinate: g/l fuel; abscissa: different investigastions)

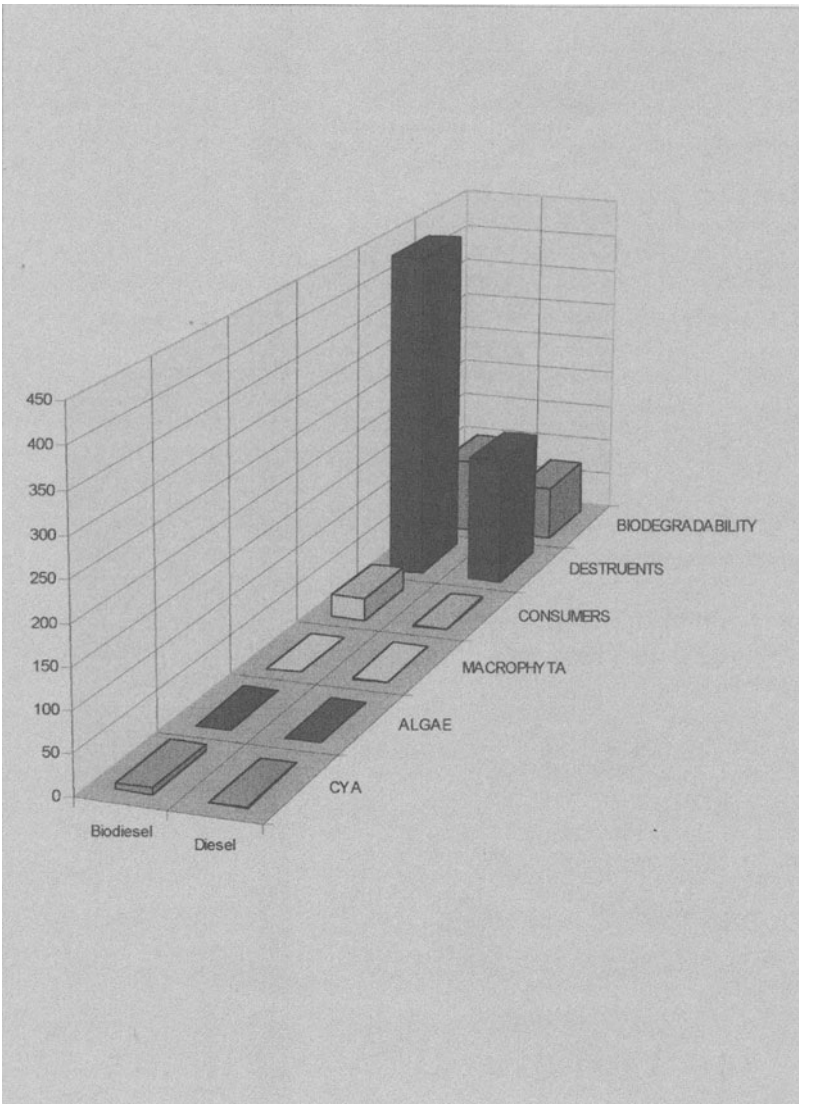

Diagramm 4. Averages from EC10 data (Cyanobacteria, algae, macrophyts, destruents) and EC50 data, which were relevant for the ecotoxicity assessment (ordinate: g/l fuel)

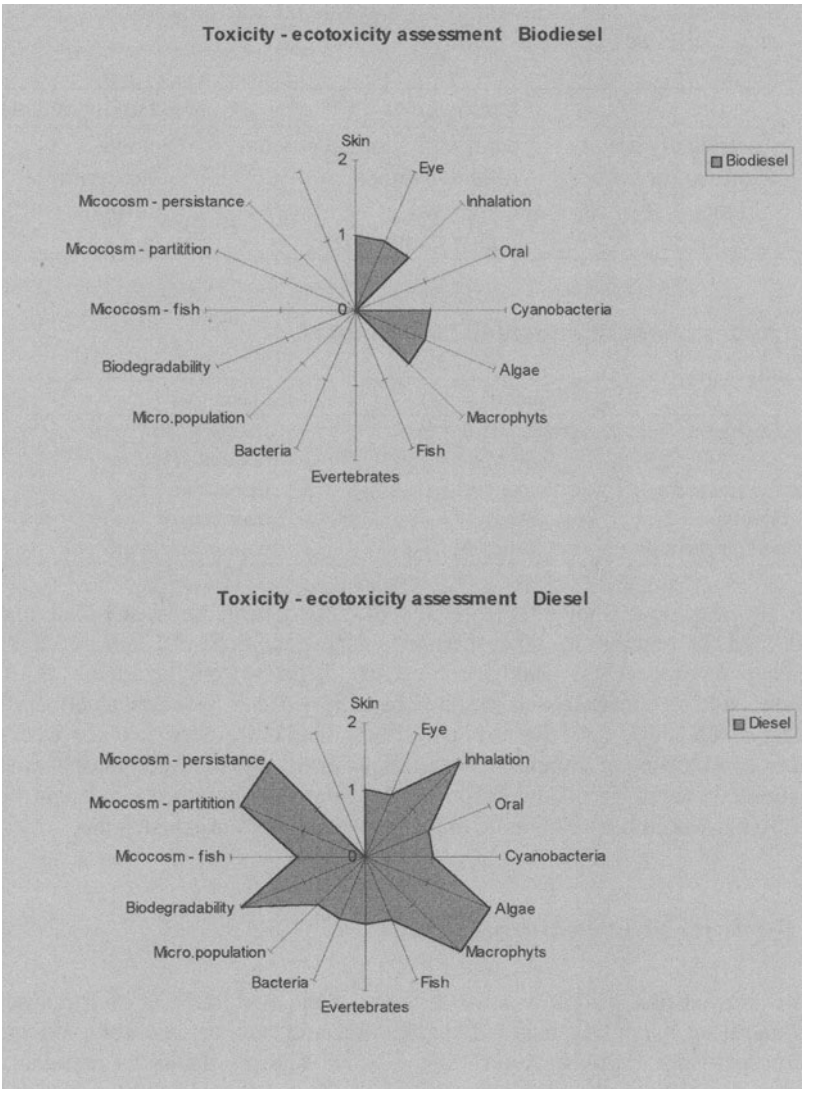

Diagram 5. Scale for the toxicity-/ecotoxicity- and biodegradability referring to Biodiesel and petroleum diesel fuel

In diagram five the results are presented in combined way. One can see that petroleum diesel fuel does not appear to be more toxic than Biodiesel in some cases. In diagram five higher index stands for higher toxicity/ecotoxicity/ and lower biodegradability. The following chart explains the meaning of the scale for toxicity-/ecotoxicity- and biodegradability.

Scale	Toxic	Ecotoxic	Biodegradable
0	practically not	commonly not dangerous	good
1	little to moderate	little dangerous	moderate
2	highly	dangerous	difficult
3	extreme	highly dangerous	not

General assessment of effects in environment

	Biodiesel	Diesel
Toxicity assessment	practically not toxic to little toxic	little toxic to moderate toxic
Ecotoxicity assessment	little dangerous	dangerous
Biodegradability	very good	moderate
Environmental behaviour	not so problematic	more problematic

These investigations showed the effects of both petroleum diesel fuel and Biodiesel and in relation to other certain substances, they did not seem as hazardous to environmental. But if one recalls pictures from accidents of oil tankers, we have to conclude that the the chemical - eco-/toxicological effects - seem to be much lower than the physical effects which are persistent for weeks and lead to cruel killing of animals. The reactions of the aquatic Spermatophyta at coastal shores covered by oilfilm could not be found in literature but it can be imagined that it would be problematic to the growth of these organisms too.

Need for further investigations

Results showed that we know something about the acute effects of Biodiesel and also diesel on mammals, fish and evertebrates and also about chronic effects on microorganisms, cyanobacteria, algae and higher plants. Preliminary conclusions could be made on the behaviour of Biodiesel and petroleum diesel fuel in a microcosm, but this information is still insufficient to assess the hazard of both oils on aquatic or terrestric environment.

Authorities often have no problems when licensing the handling, transport, storage or sall of the Biodiesel/petroleum diesel fuel. But nobody knows what to do in case of accidential overflow of Biodiesel to the ground. The effects of mineral oil on ground water are well known and also people know that they have to carry off the soil when it is contaminated by this substance. On the other side, nobody knows anything about behaviour of Biodiesel when a spill occurs.

Therefore people are obliged to act the same as in cases of accidents with petroleum oil. Next steps in investigating the effects of both oil types should be the analyses of the behavior in different soil types. After leaching, the wet residue should also be tested by ecotoxicological means to determine if a chronical toxic effect remained. If there is no toxic effect, an effort should be made to determine what happened when Biodiesel residue has decomposed very fast in ground water.

No results were found concerning the cancerogenity, mutagenity or teratogenity. It can be imagined that data referring to these themes could be a next step to show people, that Biodiesel is much less problematic than petroleum diesel fuel.

Acknowledgements

I am very grateful to Dipl.Ing. M. Wörgetter and to Dipl.Ing. Dr. J. Schrottmaier for intruducing me to the world of renewable fuels and that they were such far-sighted to conduct biological investigations outside of their subject areas. Especially I want to thank Dr. Körbitz for his management of all fields of Biodiesel matters in Austria and for making available all literature, I never would be able to find it myself. And last but not least I am grateful to Mag. B. Zach and Mag. M. Vekilov for executing the ecotoxicity tests very carefully and to Mrs. Ofenböck for lecturing.

Relevant Literature

1. Acute dermal toxicity of „Biodiesel" in rats (Limit-test). Report for Bioenergie, Forschungs-, Entwicklungs- und Verwertungsgesellschaft m.b.H. Österreichisches Forschungszentrum Seibersdorf, Hauptabteilung Toxikologie. 1991
2. Acute oral toxicity of „Biodiesel" in rats (Limit-test). Report for Bioenergie, Forschungs-, Entwicklungs- und Verwertungsgesellschaft m.b.H. Österreichisches Forschungs-zentrum Seibersdorf, Hauptabteilung Toxikologie. 1991
3. Bewertung wassergefährdender Stoffe - hier: Einstufung von Fettsäuremethylester, C-Zahl> = 6 in eine WGK. Institut für Wasser-, Boden- und Lufthygiene des Bundesgesundheitsamtes, Kommission Bewertung wassergefährdender Stoffe. 1993
4. C. Birchall, J.R. Newman, M.P. Greaves, Degradation and Phytotoxicity of Biodiesel oil. Institute of Arable Crops Research Long Ashton Research Station, Centre for aquatic plant management, Reading. 1994
5. Einstufung von Gefahrenbezeichnungen nach GefStoffV 1986, BMA & S
6. Einteilung nach Toxizitätsklassen nach SPECTOR, W.S.: Handbook of toxicology. Vol.1, Saunders Philadelphia 1956
7. Gutachten über Rapsmethylester u. Dieselkraftstoff. Höhere Bundes-Lehr- und Versuchsanstalt für chemische Industrie Wien XVII, Staatlich autorisierte Versuchsanstalt für Biochemie und Schädlingsbekämpfung 1992
8. Gutachten über Öko-Diesel-Additiv Pur und Additivierter Öko-Diesel. Höhere Bundes-Lehr- und Versuchsanstalt für chemische Industrie Wien XVII, Staatlich autorisierte Versuchsanstalt für Biochemie und Schädlingsbekämpfung 1992

9. OMV AG.: OMV - Sicherheitsdatenblatt Dieselkraftstoff 1995
10. W. Rodinger, Ecotoxicological risks of Biodiesel compared with Diesel based on mineral oil. Minutes of the 2nd Activity Meeting - Workshop, Vienna, 1995
11. W. Rodinger, Ecotoxicological risks of Biodiesel compared with Diesel based on mineral oil. Proceedings of the international conference on standardisation and analysis of Biodiesel. Bundesministerium für Land- und Forstwirtschaft, Bundesministerium für Wissenschaft, Forschung und Kultur, Bundesministerium für Umwelt, Fair - European Comission, Altener - Alternative Energy Programm der EU, Generaldirektion Energie, International Energy Agency Liquid Biofuels Activity, Bundesanstalt für Landtechnik. 1995
12. W. Rodinger, Umweltverträglichkeit von Rapsmethylester im Vergleich zu Dieselkraftstoff. Symposium „Rapsmethylester" Kraftstoff und Rohstoff, 1992
13. L. Roth, Wassergefährdende Stoffe. ecomed, Landsberg, München, Zürich, 1996.
14. Soydiesel - Biodegradability and Toxicity - Technic Information. Midwest Biofuels Inc. 1985
15. R.G. Weyandt, Ökologische Bewertung durch Umweltverträglichkeitsprüfungen. Institut Fresenius, Abt. Umweltmikrobiologie. Not published

Discussion of Session 2: Toxicological and Environmental Aspects Related to the Use of Plant Oils as Fuels

Presentation by **J Mauderly**: *Health Issues Concerning Inhalation of Petroleum Diesel and Biodiesel Exhaust.*

F Adlkofer:, Dr Mauderly, you said that you don't think health effects might be the limiting effects in introducing Biodiesel. I think that is absolutely correct. The question is: what about a lower risk resulting from Biodiesel as compared with fossil diesel? Would this not be a reason for introducing it, to improve the economic situation?

J Mauderly: Logically, yes. But politics are not always logical. If the question is posed differently and one asks: if all diesel engines in the world were running on Biodiesel rather than petroleum diesel, would this be a better world? It's difficult to say, but probably, yes. The next question is: how much are you willing to pay for this better world? And my answer is: I'm not sure, I'd have to ask my colleagues, and how much money are we really talking about? The answer really depends on how concerned one is about the risk of petroleum diesel exhaust. This is more political than scientific, so I cannot give a very good answer.

Presentation by **J Krahl**: *Biodiesel Exhausts Emissions and Determination of their Environmental and Health Effects.*

G Oberdörster: What kind of cell is the L929 cell that you used for toxicity testing?

J Krahl: A mouse fibroblast cell line.

G Oberdörster: How do the results compare with other cell types that are used for toxicity testing?

J Bünger: We did the same test with L 132 cells, an established embryonic lung cell line, with the same result.

J Mauderly: With respect to mutagenicity, it was not clear, whether you meant specific mutagenicity, i.e. mutagenicity per microgram of extract, or total mutagenicity, i.e. mutagenicity per hour of use of engine or per mile travelled or something like that? What were the units?

J Krahl: The unit was the volume of exhaust. The exhaust volume is nearly the same whether an engine is run on diesel or Biodiesel, although the mass can be completely different. Exhaust gas from RME, as in the example of the French tractor, can have double the mass of fossil diesel exhaust. We always took a volume and extracted a volume.

J Mauderly: So this would be a comparison of mutagenic activity per unit of equivalent engine use.

G Oberdörster: The particle size distribution measured by an impactor showed a shift towards larger particles. Did you make any electron micrographs to see if these particles consisted of agglomerates. If so, what kind of agglomerates were they, and what were the smallest particles?

J Krahl: This is being done currently in Magdeburg. The electron micrographs show the agglomeration of Biodiesel particulate—this is the first result—to be higher than with fossil diesel. Thus we find the same tendency with microscopy as well.

G Oberdörster: So the primary particles may be of the same size between the two exhausts, but Biodiesel exhaust agglomerates more easily.

J Krahl: Yes.

F Adlkofer: May I return to the mutagenicity related to the volume of exhaust? If you need more Biodiesel to go a certain distance because it is less energetic than fossil diesel, Biodiesel might therefore generate a higher volume, and the total mutagenicity resulting from Biodiesel might be even higher than with fossil diesel.

J Krahl: The energetic fuel consumption is the same for Biodiesel and diesel. The volumetric consumption is 8 % higher and the gravimetric consumption is 13 % higher, whilst the differences in the mutagenicity tests between diesel and Biodiesel are larger than 13 %.

J Schell: Do you have any recommendations for the plant breeders on the basis of your data?

J Krahl: The transparency showed the technical procedure—measurement, assessment, technical consequences. At the moment we are between the first and second steps and we are not sure what to recommend.

Presentation by **P Eckl**: *The Mutagenic Potential of Diesel and Biodiesel Exhausts.*

G Oberdörster: My question concerns the dosing of the hepatocytes. You expressed the dose with respect to exhaust volume. What is the mass dose given to

your cell system? For 50 l exhaust volume, for example, how much of that is particulate and organic mass?

P Eckl: The particles have to be extracted when we perform the assay. We get a total sample volume of some 10 ml, corresponding to about 1-2 m^3. Recalculating for 1 l, gives a maximum of about 50 ml sample volume or so. For the dose-response curves, up to a maximum of 5 % was used in the test system, i.e. 5 % of the volume, making it picogram per 500,000 cells.

J Connemann: Could you describe the ester that was tested? If, for instance, it contained considerable glycerol, the exhaust gases will have very high content of acrolein. It's very difficult to compare the different results of toxicity testing. It is important to know what sort of Biodiesel being tested.

P Eckl: That is why we compared directly the Ames Test and the hepatocyte assay with the same sample, so as to have the same concentration of substances present.

J Connemann: The results are very different with a high content of methanol or a high content of glycerol. The limit for total glycerol content here is 0.25. For the USA Mr D. Reece showed me values from 1.80 to 0.64. The best one was 3 times above our limit.

J McDonald: This is correct, although I have seen much better quality esters. My data are from a very pure double vacuum-distilled ester. However, I have also participated in a field trial in an underground mine. I had to return 2,000 gallons of fuel to the supplier because of contamination with glycerol, glycerides, and water. Ethanol and methanol, moreover, are aldehyde precursors, and in diesel combustion they could combust partially to form formaldehyde and acetaldehyde.

H Prankl: In reply to Mr Connemann. The fuel was produced at our institute. The fuel specifications met DIN standard (except neutral number and water content).

Presentation by **J McDonald**: *Biodiesel: Effects on Exhaust Constituents.*

J Mauderly: If I understood correctly, you said that in your studies the greater soluble organic fraction of soot was attributed almost exclusively to a very narrow set of unburned fuel constituents, linolenic, linoleic, oleic acid esters.

J McDonald: Yes. Of the mass of the organic material collected on the filter, 55 % was contributed by those compounds.

J Mauderly: Given other data, do you think that is characteristic?

J McDonald: Yes, and it may have a lot to do with the variability in particulate matter emissions from engine to engine. Under some conditions organic material

might exit in the vapour phase, under other conditions in the particulate phase, depending on the dilution tunnel and the dilution ratio used. It also depends on the fatty acid profile of the Biodiesel fuel used.

J Mauderly: Would you then predict that if the plant and fuel processors were clever enough to arrange the hydrocarbons on either side of that peak, the organic fraction would be markedly reduced?

J McDonald: Yes, if it were arranged on the lower carbon number side of that peak. A careful examination of the toxicity of methyl esters and the particle phase is warranted. If the material is relatively benign, what difference does it make? It makes a difference from the standpoint of regulated emissions, because regulated emissions are defined by a temperature, 52°C, dilution with air, and deposition on a specific type of filter. Dilution ratios typically used in laboratories are between about 10:1 and 15:1. In view of this and a comparison with dilution in ambient air, there would appear to be significant differences between a particle in a laboratory dilution tunnel and a particle in ambient air. Our tests in underground mines show just this. Just because organic material is present in the particle phase in the dilution tunnel doesn't mean that it will ever enter the lungs as particulate matter, because it may just evaporate once it's diluted more with air. And even if it does, it need not be harmful.

F Adlkofer: If compared with the data of Dr Eckl and Dr Krahl, your data would appear to favour the future use of Biodiesel. What is the reason for that?

J McDonald: I defer to Dr Mauderly. It is not worse with respect to indicators of potential health effects.

F Adlkofer: Did you use Biodiesel from a different source?

J McDonald: Soybean oil. The differences include, firstly, the indirect injection engine. In our experience, indirect-injection engines are much more tolerant of fuels with different physical characteristics. Those two particular indirect injection engines can operate on high percentage soy methylesters, but recent testing of a very modern direct-injection Navastar engine demonstrated that it could not run with much more than 50 % soy methylester without reliability problems. Regarding the emissions, so many factors influence the measurement of emissions results. I would need to see the error bars, to see the uncertainty level. Factors such as the fuel, compounds in the fuel, error or statistical uncertainty can influence the outcome. There have been frequent reports on carbon monoxide reductions, but carbon monoxide is not a problem with diesel engines. There are no efforts to reduce carbon monoxide emissions in general with diesel engines, because these are very low. The question arises: for those emissions that currently are a concern with diesel engines, such as PAH, are the concentrations above the minimum detection limit, and if so, are they still really low?

G Oberdörster: How old were the engines you used, how long had they run?

J McDonald: The MWM engine was brand new, but it's an older design. Many of the engines used were older.

G Oberdörster: Do emissions change with engine age?

J McDonald: The same engine design is produced today. The engine was about 5 years old, but had run for less than 100 h. The Caterpillar engine was recently rebuilt. It had run for about 1500 h, and was rebuilt to brand-new specifications.

G Oberdörster: Could you compare emissions of an aged engine with those of a brand-new one?

J McDonald: An aged engine has increased organic emissions, increased oil consumption, increased particulate matter emissions, and the oxides of nitrogen emissions decrease. There are differences: both those engines were well broken in. Do you mean a brand-new engine off the factory floor? Or a newer engine design? The particulate matter emission for the Caterpillar engine was around 0.4 - 0.6 g/kWh, which is very high. Modern engines achieve typically 0.1 g/kWh, and even this can be reduced. The Series 60 and Series 50 Detroit Diesel engines for instance, which have been tested extensively by South West Research Institute, have shown very similar reductions in total particulate matter when used with a diesel oxidation catalyst to these older style engines, although I would expect the particle size distribution from those more modern engines to be somewhat different. They may have more nuclei mode particles.

Presentation by **W Rodinger**: *Toxicology and Ecotoxicology of Biodiesel Fuel.*

G Oberdörster: What was the "inhalation" endpoint in your data?

W Rodinger: The inhalation data I found in the literature.

G Oberdörster: On Biodiesel fuel?

W Rodinger: On fuel and on diesel exhaust.

G Oberdörster: So that's combined, exhaust plus fuel.

J Mauderly: Dr Rodinger, did you see any biological endpoint in any system that gave you cause for concern? In all the many measurements there was not one in which the Biodiesel was worse than petroleum diesel.

W Rodinger: I found only one with macrophytes. The Ashton research had found Biodiesel to be more toxic to macrophytes than mineral-based diesel.

J Mauderly: What might the reason for this be?

W Rodinger: The literature suggests that there is a physical or mechanical effect which influences toxicity more than the chemical effect.

G Oberdörster: In the final part of this discussion, the major concern is questions that can be addressed to the plant bioscientists with regard to improving the product Biodiesel and its exhaust, in terms of its toxicological and ecotoxicological impact? From what has been said, Biodiesel looks very good. Particulate emissions from Biodiesel fuel were basically less toxic than petroleum diesel and there was also a significant decrease in PAH. The only increases for Biodiesel reported in different studies have been NO_x emissions and perhaps aldehydes. In the first talk, Dr. Mauderly emphasised three diesel exhaust components that should be evaluated, namely particulates, PAH and irritant compounds. We will discuss the toxicology first, and then move to the ecotoxicology and try to formulate specific questions for the plant scientists.

E Garshick: It might be relevant to review the potential health effects in humans, some of which were addressed by Dr Mauderly. Odour and irritant effects were mentioned. The relevance of these will depend on the presence of irritants such as acrolein, and given the variable fuel glycerol content, the presence of NO_2. Respiratory symptoms such as cough, phlegm and wheeze may occur in exposed individuals. Under certain conditions the fuel and engine operating conditions may also be important. Studies of acute exposure to fossil fuel diesel exhaust have shown pulmonary function changes in exposed individuals, although not consistently so. This might be relevant for individuals exposed by operating vehicles powered by Biodiesel fuels. For fossil fuel diesel, there is little or no information on the effects of chronic exposure on pulmonary function. There are no long-term longitudinal studies. We should consider this if a new industry is to be developed, and start to make such measurements in individuals who may be exposed subsequently on a long-term basis. It has been suggested that bladder cancer can develop in workers exposed to PAH such as occurs in fossil fuel diesel. However, the epidemiologic literature linking fossil fuel diesel exhaust to the occurence of bladder cancer is not conclusive. Motor vehicle operators do show an increased incidence, but it's unclear whether this can be attributed to diesel exhaust exposure since exposure in these studies has not been well characterized. Epidemiologic studies have also suggested that there is a small increase in lung cancer risk in workers occupationally exposed to diesel exhaust over many years.

Speaking as an epidemiologist and physician, a lesson relevant to the Biodiesel industry is to characterize emissions thoroughly since emissions can be influenced by the specific fuel being used. It is important to determine an appropriate emission marker to be used for environmental sampling. In studies on fossil fuel diesel exhaust one factor limiting the interpretation of epidemiologic studies is the lack of a marker specific for diesel emissions. Recent work has indicated that elemental carbon is a marker of emissions for fossil fuel diesel exhaust. This might be an appropriate marker for Biodiesel based exhaust emissions, or biomonitoring using new technology may be possible. In the Biodiesel industry relatively few individuals are exposed as yet. It would be useful to start prospective studies in workers with the extent of exposure well-characterised in

order to study the potential health effects. Finally, surveys assessing respiratory symptoms and pulmonary function should be conducted among the individuals whose exposure is well characterised. These surveys must be carried out over many years, particularly in view of the long latency of cancer, which is usually more than 20 years for lung cancer. A potential major confounder in any study of respiratory function in an occupational cohort would be smoking habits, and I would urge that smoking habits be considered prospectively.

G Oberdörster: Such studies are certainly important, but at the moment the population in which such studies could be performed is not large enough. As important as such studies are, until there is a large enough cohort we should to focus on characterising the toxicity and carcinogenicity which might be involved with Biodiesel. Even if, for instance, particulate emissions are lower from Biodiesel, the effects need not necessarily be less. However, our information to date suggests that Biodiesel exhaust has a lower mutagenic, carcinogenic and potentially also irritant potency. However, we cannot be certain until studies have been performed in a mammalian species.

J Mauderly: Overall nothing has suggested strongly that health concerns will limit the use of Biodiesel. This is a very broad statement, but probably a good starting point. I would encourage the plant scientist or the fuel processor or the engine manufacturer as follows: if a very narrow family of hydrocarbons contribute the majority of the organic mass in exhaust, then something may be gained by trying to reduce the content of that particular family of compounds in the fuel. Why? Even if this organic material is not strongly mutagenic, if it contributes up to 50 % of the total organic, which may be 20 % of the particle mass, then reducing it would reduce particle mass concentrations even further. With respect to work that ought to be done on health effects, there is a difficulty in that information has already been obtained from many different kinds of fuels and engines. For a health or laboratory scientist beginning a more definitive evaluation it would be highly desirable if some typical condition were to emerge as representative. Will there be a typical fuel or a most typical engine? Until the industry progresses to the point where there some uniformity, biological results will vary tremendously depending upon the conditions.

Assuming that "typical" exhaust generation conditions could be determined, what then? One gap in our current understanding that would be very straightforward to resolve would be a simple animal inhalation study to compare the irritant response. Although extrapolation from animal studies to humans is not without problem, comparative studies of a certain dilution of petroleum diesel exhaust or a Biodiesel exhaust could still be performed in rodents to see whether the response to the total diluted exhaust—the vapour phase and the particle phase —is really noticeably different. Such a study would be neither time consuming nor expensive nor hard to do.

G Oberdörster: Something should probably be done in a mammalian species to compare the two exhausts. Separating the gas phase may not necessarily be immediately necessary, but comparative data with respect to toxicity, inhalation

toxicity are needed to establish whether Biodiesel may be of less concern than petroleum diesel, or how they differ. The point about a standardised fuel is a very good. A DIN norm for Biodiesel has been discussed: will it be good enough to have a standardised fuel? Can this be a basis for testing purposes?

J McDonald: That is my suggestion. Diesel fuel, petroleum diesel fuel, is by no means homogenous. One thing that has been done in testing petroleum diesel fuels and fuel additives has been to standardise a test fuel with tighter specifications. This is done for certification purposes all over the world. Both the USA and the EU have standardised test fuels with specific properties that are representative of a typical fuel, but allow much closer comparison of different exhaust properties and much more repeatable results. I would thus recommend a test specification for a test fuel that is typical and is more stringently controlled than the biological material obtainable, which can vary considerably from season to season and from processor to processor.

H Klingenberg: To return to the health effects on humans and the threshold Dr Mauderly mentioned. The air quality values in a street with dense traffic and quite a high number of diesel cars, show some 10 $\mu g/ m^3$. If the fleet were to have 20 % Biodiesel cars, an optimistic value, the concentration would fall to about 9 $\mu g/m^3$. With the impact being far below the effective threshold, changing the fuel will have practically no effect on humans.

G Oberdörster: That is certainly correct for the overall exposure situation, but if the Biodiesel is used specifically in certain areas where people are exposed only to Biodiesel, this would be different.

H Klingenberg: That is a very limited application. For instance in mines, where diesel engines have to be used, the particulate concentration can be reduced, but it must be realised that there are also other effective particulates that are present in far, far higher concentrations than diesel particulates.

J Mauderly: If I may respond briefly. Professor Klingenberg referred to my use of the term threshold. I want to make clear how I was using that term, namely in relation to the animal studies, in particular the rat for which there seemed to be a threshold of some sort for both the lung tumour response and the non-cancer lung irritation response. I do not know if threshold exists for human responses, and when discussing environmental exposures at the concentrations mentioned discussing, this is very relevant. In this situation it is not simply exposure to diesel exhaust only, but exposure to ambient particulate material, which is a far broader issue. Within ambient particulate material, diesel, Biodiesel would be just one of many constituents. The threshold issues would be quite different at those concentrations.

H Klingenberg: I am aware of the threshold, and I was going to ask about the current state of the discussion. I have followed the question of thresholds for many years and indeed it is not known if there is one for humans. Referring to the

particulates again, a concentration change of only 1 µg/ m^3 is 10 %, and considering the other particles present at concentrations far higher than that from the diesel cars, my argument still holds true.

G Oberdörster: There may be another argument of importance: The US-EPA is trying to introduce a so-called 'Fine Particle Standard'. Within the fine particle mode there is the nucleation mode of ultrafine particles, which may be very important. The mass of this mode is not so relevant because it is very, very low, so even 1 µg represents a large number of particles.

J Schell: Molecular breeders can achieve something only if the task is clearly defined. It has been suggested that most of the organic particulate matter originates from only a few organic products. Can these be identified?

J McDonald: In my studies they have been identified. They were primarily methyl esters and oleic and linoleic acids.

J Schell: This is where the molecular biologists and breeders in general could do something about it.

J McDonald: I am aware of this. However, when discussing points of no return and NO_x emissions the ignition delay effect must be considered. Evidence from research in the USA shows that going to exceptionally short ignition delays with exceptionally high cetane number fuels may actually increase oxides of nitrogen emissions. Since there has been very little combustion work done with Biodiesel it is not clear if this is also the case with Biodiesel fuel. It is conceivable that the increase in cetane number above 60 in Biodiesel fuel might be contributing to the detrimental effect on oxides of nitrogen emissions. Thus it's not necessarily a good idea to go to even higher cetane number fuels.

G Oberdörster: You mentioned specifically a change in condensation and absorption characteristics with respect to the particulate output.

J McDonald: We did some initial modelling of the condensation and absorption characteristics.

G Oberdörster: What are the implications for specific fuel constituents?

J McDonald: Carbon number. Again carbon number. Lower molecular weight fatty acids would be less likely to condense and absorb and other things are affected also. The degree of saturation and branching of the molecules also affects condensation and absorption characteristics.

G Oberdörster: So lowering the carbon chain number is a desirable trait?

J McDonald: Yes, but organic material is not the entire problem. Methyl esters appear to be fairly benign, and although they may condense and absorb in EPA or

EU-style laboratory dilution tunnels, there is little or no evidence for their condensation in ambient air. One might ask: why go to all the effort of modifying the fuel if the organic material in the ambient does not condense or absorb to form particulate matter at the high dilution ratios present in the atmosphere.

G Oberdörster: So you see no need to change anything at the moment?

J McDonald: No. If the problem is meeting a particular particulate matter standard, this has already been achieved compared with petroleum diesel fuel.

G Oberdörster: What do you call a standard?

J McDonald: In the EU it is 0.15 g/kWh. If a particular engine already achieves that with Biodiesel, or is no worse than with fossil diesel, it doesn't need to be reduced.

G Oberdörster: This is a general standard for emission, but it may not necessarily mean that it wouldn't be good to reduce it further if possible.

J McDonald: It's possible, but if it's not condensing into particulate matter in the ambient air, is it a hazard to health?

W Rodinger: Particulate matter from fossil diesel is always being compared with that from Biodiesel. In principle it is incorrect to compare gravimetric values: we conclude that the value is higher, and that the effect is worse. Investigations have shown that the composition of the particulate is completely different and the effect of the particulate may be different. Comparing the fuels on the basis of gravimetric value is like comparing oranges with apples. There has also been a proposal for shorter fatty acid chains in the fuel. If this were done, I suspect that aldehyde emissions would increase, because the aldehyde emissions may be connected with the ester group in the fuel. Shorter chains mean more molecules for the same heating value, and so the emission of toxic aldehydes may increase.

G Oberdörster: Your comment on particles is correct; all particles are not necessarily the same. Although masses are not always the same, what else can be used? Toxicology employs other dosimetric indices such as surface area. This is very important for agglomerates of very fine particles for which surface area could be very large; this has an impact on the effects. However, without animal studies it is hard to predict what the effects of Biodiesel will be compared with petroleum diesel. Toxicological studies in that regard are necessary. It may be predicted that reduced particle emissions by mass are beneficial, but this is not certain.

P Walzer: Many of the results relating to particulates and NO_x have been gained from today's not very advanced diesel engines. Future emission legislation will require that diesel particulate emission be almost as low as that of gasoline engines and that NO_x be reduced considerably. To achieve this, the same improvement step that was made in emissions from 1975 till today must be

repeated by the year 2002/2005, especially in the USA. Diesel engine technology is developing in the direction of high-pressure, multi-injection and pre-injection, and even to control of combustion process. NO_x will be reduced by very high degree of exhaust gas recirculation. All this will change the emission constituents. So in a certain respect, we have been discussing a status which is already past. For instance, it was suggested that the diesel should perhaps have a pre-chamber etc. This is, in other words, attempting to choose an engine according to the fuel. Engine development is however proceeding in other directions. It would thus be preferable to take such a very advanced diesel engine and run it on Biodiesel and so determine the direction of changes in emissions. Perhaps Biodiesel can even help under these conditions to meet future emission legislation requirements.

G Oberdörster: This problem occurs constantly. Whenever research is undertaken, the engine is already outdated.

P Walzer: Outdated is the wrong term, the direction of engine development is changing, and if Biodiesel is to be a fuel for all engines, the combustion processes of these advanced diesel engines must be studied. This is another area where information is inadequate.

F Adlkofer: Dr Walzer is implying that Biodiesel should be treated in the same way as fossil diesel, because the problems might be solved by engine technology. The outcome for the environment and for humans exposed to this diesel would be exactly the same if the problems are solved by changing the engine. The second point is NO_x/NO_2. Plant biologists can't do much to reduce NO_x and NO_2. It comes from the air and is created when Biodiesel is burned.

G Oberdörster: Your point was that when an improved Biodiesel engine is available, the exhaust constituents and their effects should again be characterised.

F Adlkofer: The consequence is that there is no advice to be given to the plant biologists at present. There are possibilities in changing the engine, and that is valid also for the normal diesel. It means that both could be treated similarly, including the NO_2/NO_x problem. That would be solved by engine technology in the same way as with fossil diesel.

P Walzer : I cannot predict how Biodiesel will perform in such a high-pressure injection, high exhaust-recirculation environment. Hopefully better, but we should base our first research on these results. The first and urgent step is to investigate Biodiesel in such a modern engine.

J McDonald: To some extent such investigations have already been made in the USA, at least using soybean-based diesel fuel, mostly in blends with petroleum diesel fuel. To my knowledge there have been no studies on high-pressure injection systems and highly-boosted direct-injection engines with either pure or a high percentage of rape methyl ester.

G Oberdörster: There are data for soybean methyl ester?

J McDonald: For blends, and limited data for pure soy methyl ester on Detroit Diesel Series 50 and Series 60 engines, and I think also on the Caterpillar 3406E. These engines are pretty much state-of-the-art as far as heavy-duty engines are concerned.

G Oberdörster: What do the results suggest?

J McDonald: With respect to the carbon fraction of particulate matter the results are fairly similar. Carbon particulate matter is reduced more or less in parallel with the oxygen content of the fuel, so blending Biodiesel with petroleum diesel reduces the carbon fraction and tends to increase the organic fraction.

G Röbbelen: Dr McDonald, you stressed a test fuel. In what way is a test fuel different from a norm? The test fuel also has to be defined, one simply needs the details of the qualities present. Whether this is a test fuel or a written norm, is this different? If not, a standardised blend may be more realistic and immediately useful than a norm. But otherwise a norm is as good as a test fuel, isn't it?

A Schäfer: No. Consider density, for diesel purposes, as an example. EN 590, the existing diesel standard in the EU, demands densities of 820–860 kg/m^3. In comparison, the reference fuel that must be used to certify our engines, for example, allows just 835–845, i.e. just one-sixth of the permissible range of densities found on the market. This enables engine-typical effects to be distinguished from fuel-typical effects. All properties of a reference fuel have to be defined far more stringently than the normal standard for market use.

J McDonald: Consider the current range of fuel sulphur contents. Testing fuel additives in the U.S. requires a sulphur content of 0.05±0.0025 %; engine certification testing requires a sulphur content of 0.03–0.05 %. There is a range of aromatic contents that has to be adhered to. For diesel fuels, for certification fuels, there is a whole range of much tighter specifications than are required for over-the-road vehicles. A good example would be the use of on-highway diesel fuel for certification testing in the USA. Everyone would use zero-sulphur fuel—a legal on-highway fuel—that would result in much lower particulate emissions, and would show the engine to be somewhat greener than it really is.

P Eckl: From a toxicological point of view, Biodiesel seems better than diesel, except for few discrepant results like mine. But uncertainties remain, in particular because there are no in vivo data and hence no direct comparison can be made with fossil diesel data. Thus there is a demand for more research in this area. Furthermore, from a toxicological point of view, if the engine designers or fuel designers agree that longer-chain, or shorter- or branched-chain fuels are preferable, the toxicologist can evaluate afterwards the advantages or otherwise. The fuel must first be designed, then it can be evaluated and consequences drawn.

G Oberdörster: That is, both the fuel and the improved engine are necessary before toxicological testing can be started.

F Adlkofer: This direction presents a big problem. It is really not known which substances in Biodiesel exhaust are the most dangerous, nor is it known which substances have to be removed from the exhaust to make it safe for the environment and exposed animals and humans.

G Oberdörster: General suggestions are possible. It's always beneficial to reduce particles as far as possible, similarly PAH and irritant compounds. However the extent to which this is necessary is unknown.

F Adlkofer: These principles are without doubt correct. Although a little is known about these substances, their role in diesel exhaust toxicology is unknown.

G Oberdörster: Because there are no in vivo data on Biodiesel toxicology.

F Adlkofer: If we look at normal diesel?

G Oberdörster: The rat studies suggest that particles are in all likelihood the culprits for induction of the tumours in the rat. PAH is not needed to induce tumours in rats with high exposure concentrations.

F Adlkofer: This is exactly the problem. Whether PAH plays a role at all in these animal studies in which lung tumours were caused by diesel exhaust is not known, the particulate matter has been identified as the culprit. Extrapolating to the present situation, should we recommend that the plant biologists do everything possible to reduce particles? We don't even know the difference between particles from fossil diesel exhaust and those from Biodiesel exhaust.

G Oberdörster: This is correct. The present dilemma is that we don't know what should be recommend or even what questions should be asked.

J Mauderly: To summarise, there are limited data on the biological activity of Biodiesel exhaust. Importantly, the biologists are not waving a flag and saying there is something terribly dangerous here. This being so, only one clear recommendation can be made to the plant biologists: make plants that make more oil, and make it economically feasible. When a viable industry has been established, and fuels and engines become more standard, toxicology can be revisited.

G Oberdörster: In short, the plant biologists must increase the crop yield.

Session 3:
Contribution of Plant Breeding for Making Biodiesel Rapeseed a Realistic Proposition
(*Chairman: Jeff Schell*)

Contribution of Plant Breeding for Making Biodiesel Rapeseed a Realistic Proposition

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Introduction

In particular in Germany, many general rather negative conclusions were reached regarding the possible social and industrial impact of plant biotechnology. Presently many of these conclusions are in urgent need of reevaluation. Since the impact of plant biotechnology is presently reconsidered broadly, the same should be done with regard to „Biodiesel".

Indeed, the conclusions regarding the environmental and economic desirability of developing an agriculture geared to the production of „Biodiesel" need to be reassessed. „Biodiesel" is the „catch word" for the use of plant oils as a renewable source of fuel for motorcars. It has been demonstrated that it is possible to modify the chemical properties of plant oils (via genetic engineering). There is, therefore, a need to reevaluate the desirability to try to develop crops specifically for the production of plant oils as fuels. Indeed, this is not only a matter for breeders, but one must also take into consideration that no plant biotechnical product will ever reach the market place if it does not meet economic standards or if it cannot be justified economically. Because the protection of our environment is more and more also an economical necessity, this should indeed be considered.

Another point is that we are presently experiencing an era of unprecedented rapid progress in our capacity to study, understand and therefore adapt living organisms better to the long-term needs of society, including the preservation of an environment that would make human life not only more interesting but also sustainable.

In view of this rapid progress it is essential to review supposedly sound conclusions in order to avoid potential mistakes. We did not want one more meeting in which protagonists of pro and contra discuss in a more or less convincing way the arguments and facts that led them to their respective points of view. Instead it was decided to have a meeting in which one would not only review the state of the art but also attempt to answer the question „What needs to be done to develop plant oils as fuels and improve their chances in the market place?" The main question for research and decision makers would then be: „Can plant biotechnology contribute to achieving these goals?"

Therefore, economical, technological, ecological and toxicological aspects were illustrated and discussed:

In a first session organized by Professor P. Walzer from Aachen, different speakers tried to answer the fundamental question of what should be done from the point of view of the motor-industry.

A second session organized by Professor G. Oberdörster from Rochester (U.S.), was devoted to a discussion of the environmental and toxicological aspects relevant to the use of plant oils as fuel.

A third session was organized by Professor J. Schell and Dr. N. Martini (MPI Köln) and was intended to illustrate what breeders (traditional and molecular) can and have achieved with rapeseed, thus demonstrating that biotechnology can vastly improve the yield potential of rapeseed in general and with regard to modified fatty acid composition of oils, in particular.

At the end of these presentations Dr. N. Martini of the MPI in Cologne reported on recent progress achieved in the genetic engineering of rapeseed (*Brassica napus)* for oil yield and quality, with toxicological and motor-technical aspects of Biodiesel in mind. In this presentation the potential of plant genetic engineering to answer the needs as defined by motor-industry, environmental and toxicological experts, was discussed.

These presentations were followed by a Round Table and General Discussion.

Breeding of Hybrid Rapeseed

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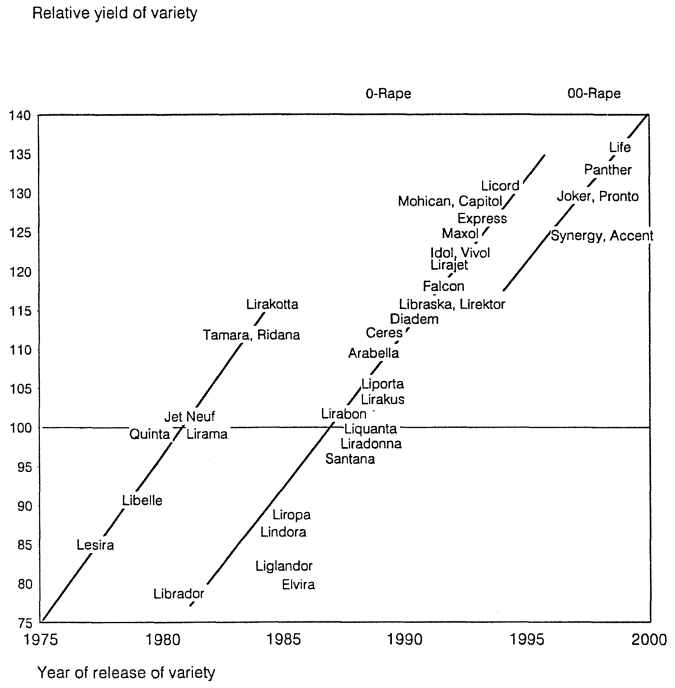
Increasing demand predominantly of vegetable oil, but also of protein for utilization in both food as well as industrial uses leads to the conclusion that there is a general need to improve productivity of rapeseed. This implies an increase in rapeseed yield per area unit (kg/ha).



Source: BSA Hannover

Figure 1. Increase of yield in winter rapeseed in varieties released in successive years in Germany and representing the 0- and the 00-quality group as well as hybrid varieties. The line of reference yield (= 100) is based on the mean of the respective standard varieties of the Bundessortenamt (BSA), Hannover.

Since of the currently only 1 to 3 % annual yield increase approximately 50 to 60 % is due to genetics, fundamental changes in breeding for yield improvement are required to boost vegetable oil production. This bears in mind that the perspective for a general extension of the production area is very limited.

There is evidence that the use of hybrid mechanisms in rapeseed breeding can contribute to better exploit the yield potential in available rapeseed germplasm.

The evaluation of yield of winter rapeseed varieties in Germany from 1975 to 1996 makes evident that the addition of each quality trait affected the yield level negatively and a decade of permanent backcrossing and selection for high yields and good disease resistance was required to recover the same yield level as that exhibited prior to the introduction of both zero-erucic-acid and low-glucosinolate traits.

Figure 1 shows the rise of yield of the zero-erucic-acid varieties (1st line left in figure 1). The first double-low varieties again yielded less than the best zero-erucics, but only 5 years after the release of the first double lows this category reached the same yield level (2nd graph in the middle). At present - after a transitional period of first hybrid releases the actual hybrid varieties clearly outyield the open pollinating varieties (3rd graph right).

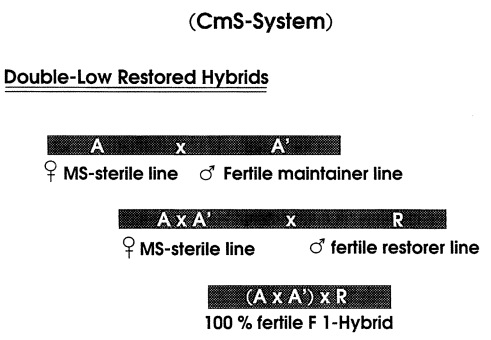

(CmS-System)
Double-Low Restored Hybrids
A x A'
♀ MS-sterile line ♂ Fertile maintainer line
A x A' x R
♀ MS-sterile line ♂ fertile restorer line
(A x A') x R
100 % fertile F 1-Hybrid

Figure 2. Breeding schedule for fertility restored rapeseed hybrid varieties by use of cytoplasmic sterility and restoration

To produce hybrid rapeseed varieties a hybrid system comprising male sterility and restorer genes is needed according to the demonstrated scheme (figure 2). Various hybrid systems were detected and/or developed, which I like to refer to and to comment on (table 1):

Table 1. Important systems of pollination control in rapeseed hybrid breeding

System	Origin	Advantages/Disadvantages
Ogura	Radish	A - stable male sterility - sufficient genetic variability for all components D - close genetical linkage between restorer- and glucosinolate gene
Male sterility Lembke (mSL)	Rapeseed	A - sufficient genetic variability at restorer side - male sterility relatively stable D - narrow genetical base within maintainer lines - expensive maintainer breeding necessary
PGS	Genetec	A - all hybrid components available - hybrids with herbicide resistance D - hybrid components only producible by genetec - production of components/seed multiplication and use are defeated by gentec law - not many practical experience
Polima-System	Rapeseed	A - maintainers and restorers easy to produce D - system only usable in spring rape (high temperatures at flowering necessary for stable male sterility) - seed production difficult
Selfincompa-tibility (SI)	Rapeseed	A - sufficient genetical variability for all components - restorer not necessary D - maintenance of SI-lines very expensive

All hybrid systems require an increase expense for seed production!

Ogura	high glucosinolate content (tight genetic coupling to the restorer gene)
Male sterility	Lembke (mSL) no problems on restorer side, maintainer breeding expensive
PGS-system	all hybrid components available, lack on practical experience
Polima	maintainers and restorers easy to produce, male sterility not always stable in various environments, seed production difficult
SI	high genetic variability of components useable, maintenance of SI-lines difficult

The positive impact of hybrid breeding in rapeseed can be shown through experimental data for morphological characters, growth and yield. The following morphological characters clearly show an increase in hybrid cultivars (table 2):

Plant length by 19 %
Diameter of stem basis by 11 %
Number of asserted lateral branches by 14 %
Number of pods at main stem by 16 %
Number of pods on first lateral branch by 63 %

Table 2. Morphological characters of 10 restored hybrids and their parental lines - 1994 -

Average of	Yield Parameters: Plant height at the end of flowering (cm)	Stem diameter (cm)	No. of branches	No. of pods on main branch	No. of pods on first side branch
11 Parental lines	165	1,39	10,6	57,2	23,2
Minimum	155	1,23	8,9	52,0	18,0
Maximum	178	1,61	11,7	73,1	29,7
10 Hybrids	196	1,54	12,1	66,4	37,7
Minimum	187	1,36	9,8	59,4	31,7
Maximum	205	1,78	14,5	70,4	42,4
Increase in %	10	11	14	16	63

Results determined as average of 10 plants per line/hybrid (Location: Thüle)
Source: Deutsche Saatveredelung Lippstadt

The seed yield was 15 % higher in the average and the same applies to the oil content. Less evident are at present positive effects on standing power and disease resistance. But we have to consider that we are only in the beginning of selecting cultivars for all the respective traits.

Table 3. Yield, oil content and important agronomic characters of 10 restored hybrid cultivars and their parental lines - 1994 -

Average of	Seed yield rel.*	Oil content (%)	Vigor before winter	Vigor after winter	Susceptibility to lodging at maturity	Susceptibility to cylindrosporium on leaves	Susceptibility to general disease-infestation at maturity
11 Parental lines	102	44,5	6,4	5,3	6,3	6,4	5,9
Minimum	87	43,6	5,3	3,0	4,6	4,9	4,7
Maximum	111	45,2	7,3	6,7	7,1	7,5	7,7
10 Restored hybrids	117	44,8	6,4	5,0	7,3	5,4	6,3
Minimum	104	43,7	5,4	4,2	6,5	3,8	4,9
Maximum	127	46,2	7,2	5,9	8,1	6,8	7,5
Increase in %	+15	1	±0	-0,3	1	-1	+0,4

*= AV Controls Lirajet, Falcon, Idol and Bristol = 100
**= Scoring 1-9: 9=positive expression of character
Source: Deutsche Saatveredelung Lippstadt

Prerequisites for successful hybrid breeding is the availability of promising parental lines with high combining ability for new hybrid cultivars. It can justly be stated that high performant and healthy lines are available from the earlier phase of breeding line cultivars.

Important criteria for successful hybrid breeding constitute high yielding and disease resistant parental lines, large genetic distance and high combination ability between parental lines.

High heterotic effects can be reached by using genepools of wide genetic distance. Historically a number of distant genepools is available as shown in table 4, but their utility in hybrid breeding programmes still remains to be proven. Genetic distances today can also be analysed by use of molecular markers.

Table 4. Genepools established in rapeseed by former breeding activities

Winter rapeseed	Spring rapessed
- Mid-west German genepool (DSV)	- Middle European genepool
- North German genepool (NPZ)	- North European genepool
- North-east German genepool (SLM)	- Canadian genepool
- French genepool (Cargill, INRA) Limagrin, Rustica)	- Russian genepool
- British genepool (CPB)	- Australian genepool
- Swedish genepool (Svalöf/Weibull)	- Chinese genepool
- Polish genepool	

The dendrogram (figure 3) reveals the genetic distance between various groups of rapeseed varieties. But the dendrogram also makes clear that a considerable number of varieties cannot be allocated to the group or family to which, with reference to their provenance, they virtually belong. This perhaps may be explained by the intensive but unreflected cross breeding during the recent years, when breeders always used the best varieties available on the market as crossing parents. In the meantime and under the auspices of hybrid breeding the establishment of genepools has become a major target of rapeseed breeding.

The relevance of genetic distance for heterosis can be shown by the correlation of heterotic yield increase and the measure of genetic distance of the given parental lines. This varies between 48 to 63 according to the material used so far, which means that about 50 to 60 % of the yield increase can be explained by genetic distance.

A further chance of yield increase in hybrids consists in the use of the special combining ability. But it is too early yet to show good examples for this additional factor since we are only in the beginning of producing hybrid rapeseed varieties

The hybrid winter rapeseed varieties, which were recently registered in Germany, are well outyielding the open pollinating varieties (table 6). So we are on the right track.

The further tasks in hybrid breeding of rapeseed may be identified as follows:

1. Permanent and systematic development of high performant and healthy lines, while considering genetic distance.

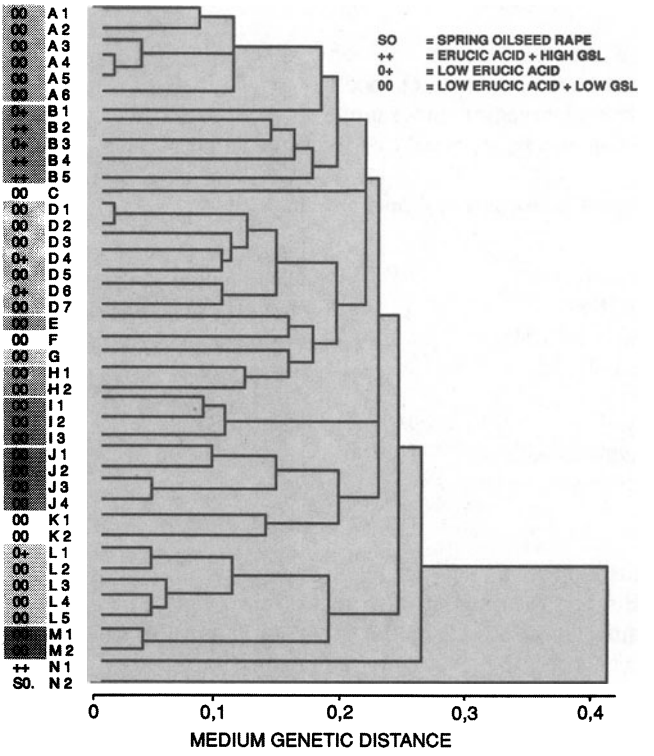


Figure 3. Dendrogram of genetic distances in 42 genotypes of winter rapeseed as developed by use of RFLP-markers. (Source: SU-Resistenzlabor Hovedissen)

Table 5. Correlation between genetic distance and relative yield of DSV-hybrid combinations (own experiments, 1995-1996)

Year	Number of locations	Number of hybrid combinations	Variation interval Rel. yield	Genetic distance	Correlation
1995	1	64	79-124	0,14-0,46	0,63
	4	64	91-119	0,11-0,43	0,56
1996	1	96	74-132	0,23-0,41	0,48

Source: Deutsche Saatveredelung Lippstadt

Table 6. Relative oil yield of registered hybrid varieties in official trials in Germany, Bundessortenamt, 1995-96

Variety	Breeder	Variety*	Relative Oilyield		
			1995 (14 Loc.)	1996 (9 Loc.)	Average (23Loc.)
Checks					
Lirajet	DSV	OP	99	105	102
Falcon	NPZ	OP	101	95	98
Express	NPZ	OP	113	103	109
Hybrid Checks					
Joker**	NPZ	RH	118	109	
Synergy**	INRA/NPZ	CHL	115	104	
Recently registered varieties:					
Panther	NPZ	RH	125	110	119
Life	DSV/CARGILL	CHL	131	114	124

*OP=Open pollinated variety; RH= Restored Hybrid; CHL=Composite Hybrid
**Varieties in 1995 not in orthogonal trials
Source: Deutsche Saatveredelung Lippstadt

2. Since glucosinolate content is tightly linked to the restoration ability in the extensively used ogu-Inra-system, extensive work has to be conducted to rapidly detect valuable restorer lines and/or to develop other, better working hybrid systems.
3. Extensive investigations are needed to guarantee complete outcrossing during seed production of rapeseed so that the best possible hybridity level can be achieved.

Pollination Control in Transgenic Rapeseed: from Concept to Market Entry of F1 Hybrids

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Introduction

The exploitation of hybrid vigour through the use of hybrid varieties is one of the major achievements of plant breeding. F1 hybrids are generally superior in reliability of yield and quality. Hybrids also offer the opportunities for the rapid deployment of resistance to diseases and pests by using dominant genes. Breeders are able to charge higher prices for hybrid seed and benefit from the exclusivity that ownership of the parent lines of a successful hybrid provide. This guarantees return sales to the seed supplier.

However, the use of hybrids has been limited to those crops for which there is an economically viable and effective means of pollination control. In addition in crops such as canola/rapeseed, an efficient method of producing hybrid seed was lacking for the successful development of hybrids. Rapeseed is therefore distributed as open pollinated varieties which represent about 5 million ha in Canada and 2.5 million ha in Europe. Due to suboptimal performance or complexities, pollination control systems have not largely been introduced in rapeseed breeding programs.

To overcome these difficulties, PGS has developed a pollination control system based on genetic engineering, called Seedlink^{TM} . This manuscript describes the design of the genetically engineered pollination control system and the development in rapeseed.

Production of F1 hybrids

Hybrid varieties are produced by controlled crosses between two distinct groups of parents, usually inbred lines. Hybrids can combine the respective traits of the parents creating a synergy so that the offspring may outperform the parents on yield, disease resistance, uniformity and general agronomic performance – a phenomenon referred to as „hybrid vigour". To produce a hybrid, cross-pollination must be ensured and the efficient prevention of self-fertilization is a key requirement. This is achieved by preventing one parent from producing pollen, that is, by making it male sterile. This female parent is then pollinated by the other

parent (male line) and subsequently harvested for its hybrid seed – the so-called F1 generation.

For seed companies, a major advantage to hybrids is that they require repeat sales. Hybrid vigour breeds out of the population over subsequent generations, thereby discouraging the use of farmer-saved seed (plant back). Because they are a superior product to non-hybrids, hybrids are in strong demand by farmers. The value of heterosis to farmers is reflected in the high prices which hybrid seeds command. Pricing to value is possible precisely because hybrids eliminate plant-back. Furthermore, because hybrids are proprietary products, their profitability is protected against the rapid erosion typical of a non-hybrid variety. The seed industry therefore shifts rapidly from open-pollinated crops towards hybrids whenever they are technically and economically feasible.

Genetically engineered pollination control system

PGS has developed a genetic hybridization system, which is a genetically engineered pollination control system (Seedlink^{TM}). It comprises two components. A male sterility component (A line) and a fertility restoration component (R line; see figure 1). The male sterility component consists of an anther specific promoter and a gene which codes for an enzyme, barnase, which disrupts normal cell activity. The barnase gene is only expressed in those anther cells which are crucial for pollen development. Barnase thereby inhibits pollen formation and renders the plant male sterile. The fertility of restoration component consists of the same anther specific promoter and the gene barstar. The barstar enzyme on its own is innocuous in plants, but combines with the barnase enzyme to inactivate it. Crossing one parent expressing the male sterility component with a second line carrying the fertility restoration component, results in the production of a F1 plant with full seed fertility.

The dominant monogenic male sterility gene is linked to a second gene, conferring tolerance to the broad spectrum herbicide glufosinate-ammonium. Upon maintenance of the male sterile line with its non-transgenic fertile counterpart, a 1:1 segregation of sterile and fertile plants is obtained in the progeny (see figure 1). In the hybrid seed production field, the herbicide is sprayed on the female rows to selectively rogue the fertiles at the seedling stage.

Male sterility on its own allows the production of hybrid seeds, but in the farmer's field half of the plants which develop from these hybrid seeds are male-sterile and the other half male-fertile. For hybrid crops which are harvested for their seed or fruit, pollination is necessary. In rapeseed, where there is inefficient pollen transfer, cross-pollination cannot be relied upon. For those crops, fertility must be restored in the hybrid seed.

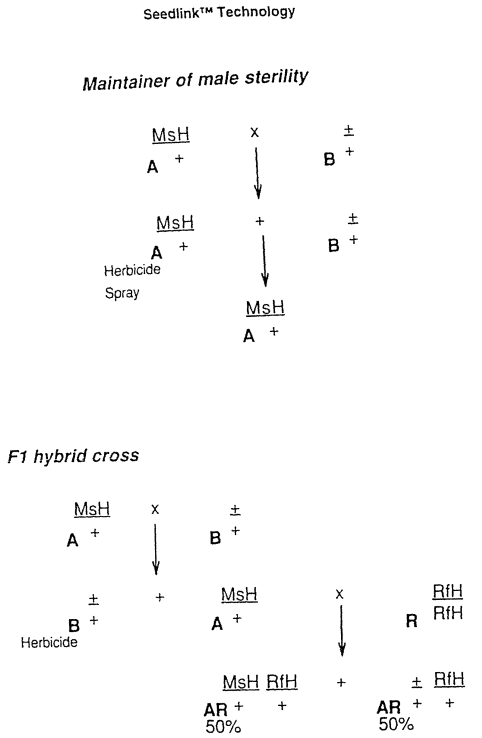

Figure 1. Schematic representation of the maintenance of male and female parents and production of F1 hybrid seed using herbicide tolerance as a selectable marker.

Selection of adequate transgene events

An essential step in the development of the pollination control system in crop species is the selection of transgenic lines with adequate insertions of the transgene in the plant genome. The transformation process generates a number of transformed lines which are all different as to the precise integration of the foreign DNA. Each line passes through different stages of evaluation until they reach a stage satisfying a number of criteria that qualify them as „elite events".
The criteria for an elite male sterility or restoration of fertility allele can be summarized as follows:

1. Stability. The male sterility or restoration of fertility has to be stable under various environmental conditions and in a range of genetic backgrounds.
2. Inheritance. Inheritance of the male sterility gene and restoration of fertility gene needs to fit the inheritance of a monogenic dominant trait and complete linkage with the herbicide resistance marker is required.
3. Herbicide tolerance. The herbicide tolerance gene linked to the male sterility gene needs to confer full tolerance to the herbicide, when sprayed at the rate required for complete removal of the fertiles.
4. Agronomic performance. Agronomic performance of seed parents and hybrids should not be negatively affected by the presence of the pollination control system.

The different criteria are selected at different stages of development in the greenhouse and small scale field trials at multiple locations.

Following a selection scheme at different levels in the greenhouse and at small scale field trials, elite loci for the male sterility gene and the restoration of fertility gene have been identified. The selected Ms and Rf allele do fulfill the following criteria:

- normal plant phenotype with respect to vegetative and quality characteristics.
- normal segregation of the herbicide tolerance phenotype (susceptible/tolerant) and of the flower phenotype (sterile/fertile).
- stability of the sterility phenotype in different environments and genetic backgrounds. Male sterile plants have been individually bagged and checked for the presence/absence of seed set.
- restoration of fertility in combinations of candidate elite alleles has been evaluated by scoring plants per plot per flower phenotype.
- agronomical evaluation of the crosses between elite male sterile and restorer alleles comprised characteristics as emergence, vigour, days to flower, days to maturity, seed yield and quality parameters (oil content, protein content, fatty acid composition and glucosinolates) in comparison with the non-transformed line.

Application of elite alleles in breeding programs

Since the early 90s, PGS has been developing three canola/rapeseed breeding programs that incorporate the Seedlink technology. In Canada, the breeding program has focused on the development of spring *B. napus* hybrids. In 1996, the first two *B. napus* hybrid cultivars were accepted for registration and commercial sale. A new hybrid should come onto the official list with a yield of 126 % of the checks in the long season zone and 118 % of the checks in the mid season.

In Belgium, the breeding program was focused on winter grown *B. napus* hybrids adapted to European growing conditions. Finally, ProAgro-PGS Ltd. (India) is developing *B. juncea* hybrids for the Indian sub-continent. Each of these breeding programs has confirmed the application of Seedlink for economic commercial seed production.

Product development and market entry

The development of Seedlink has taken a number of years to mature and time has been required for both regulatory officials and consumers of end-products to become familiar with these new technologies. The recent approval and market acceptance of transgenic tomato, canola, soybean and corn indicates that the world has indeed started to recognize the benefits of biotechnology to agriculture of the 21st century.

As part of the development of these new traits in crop species, regulatory clearance from the environmental point of view and for application in feed or food has to be obtained as well. Environmental sign off for the application of Seedlink™ components in transgenic rapeseed has been obtained for Europe and Northern America, as has been applied for (table 1: P: pending, A: approved).

Whereas fully restored hybrids have captured about 5 % of the market in Canada and Europe, it is expected that hybrids will dominate this market in the short to medium term; the limiting factor could be seed production capability. One can assume that farmers in Europe will switch to hybrids with a 10 % yield advantage over the best open pollinated varieties.

Table 1. Regulatory approval status spring 1997, Seedlink ™ technology in canola

	Environment	Food	Feed
Canada	A	A	A
EU (15)	A		
UK	A	A	A
Belgium	A	A	A
France	A	P	P
Germany	A		
Netherlands	A	P	P
US (Import)	A		
		A	A
Japan (Import)	A	A	A

References

1. M. Williams, *TibTech* **13**, 344 (1995).
2. C. Mariani, M. De Beuckeleer, J. Truettner, J. Leemans, R. Goldberg, *Nature* **347**, 737 (1990).
3. C. Mariani *et al.*, *Nature* **357**, 384 (1992)
4. M. de Block and D. Debrouwer, *Planta* **189**, 218 (1993)
5. P.G. Arnison, *PBI Bulletin* January - National Research Council Canada (1997)
6. G. De Both, *Proceedings of the 9th International Rapeseed Congress* Cambridge (1995)

Rapeseed with Tolerance to the Non Selective Herbicide Glufosinate Ammonium

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Abstract

Weed control with herbicides is essential to grow rapeseed. Glufosinate Ammonium is used as a non selective herbicide successfully in many countries for over 10 years. It conforms well with ever increasing safety standards for human beings, animals and the environment. The tolerance of rapeseed and other crop plants was achieved by genetic modification. A resistance gene (PAT or BAR) was transfered into previously susceptible rapeseed plants. This new approach allowed the development of Glufosinate Ammonium as an almost ideal selective herbicide. In cooperation with major seed companies and by own breeding programmes new Glufosinate tolerant rapeseed varieties and hybrids are developed. Data on metabolism, toxicity, residues, efficacy etc. were generated to get registration for the selective herbicide use. In addition various studies were done for safety assessments of the PAT gene and the modified rapeseed. In spring 1995 Canadian authorities granted worldwide the first approvals for the selective use of Glufosinate Ammonium (trademark Liberty) and Glufosinate tolerant (trademark and logo Liberty Link) spring rapeseed (Canola).After a successful launch in 1995 about 150.000 ha of Liberty Link Canola were grown and treated with Liberty in 1996. The Liberty Link Canola growers were very well satisfied. In a grower survey 84 % stated that they will definitely use the Liberty Link System again. In Europe registrations for Glufosinate Ammonium as a selective herbicide and for the first Glufosinate tolerant rapeseed varieties are expected in the course of 1997. The Liberty Link System will be launched in rapeseed most probably in 1998.

Introduction

Growing a rapeseed crop successfully requires, among other things, measures to control weeds. Weeds are plants which compete with the planted rapeseed crop for space, light, water and nutrients. They are undesirable wild plants as well as plants of the crop(s) previously planted. Weeds cause difficulties during harvest as

well as in the course of storage and processing of rapeseed. They reduce yield and quality of harvested rapeseeds and products derived thereof. Therefore weeds must be controlled to exploit the yield potential and assure the oil and meal quality of rapeseed. In today's conventional control methods, chemical weed control has become established worldwide as the method of choice. Other methods, especially mechanical control, are almost always inferior in economic and management terms on the farm.

No system of weed control is without side effects. For instance mechanical tillage can cause loss of valuable soil moisture and accelerate soil erosion under certain conditions. Flaming of weeds can additionally kill the accompanying fauna. The principal drawbacks of using chemical herbicides are possible residues in and on the harvested crop, an adverse impact on flora and fauna, and possible contamination of the soil, water and the air. These drawbacks are associated with any form of chemical weed control. They cannot be completely eliminated but they can be minimized. Increasing requirements for the safety of agrochemicals for human beings, animals and the environment have resulted in ever increasing registration standards of authorities. The development of new herbicides has become more and more difficult, expensive and risky.

The primary aim of modern herbicide research is therefore to develop active ingredients which meet the farmer's requirements and have the smallest possible side effects. An essential criterion for the usefulness of a herbicidal active ingredient is its tolerance by the crop(s). This is the first requirement for widespread use in crops like rapeseed.

Glufosinate Ammonium - a modern herbicide

The herbicidal active ingredient with the common name Glufosinate Ammonium meets the requirements of a modern herbicide. However, it is broad acting on all plants and cannot be used for selective weed control in most crops. So far it has been sold successfully as a non selective herbicide for many years in over 50 countries of the world. Glufosinate Ammonium has proven its favourable ecological and safety properties and is regarded as one of the most attractive herbicides.

Since genetic engineering succeeded in transferring a gene coding for resistance to the herbicide Glufosinate Ammonium into plants it was logical to develop it also for selective weed control in major agricultural crops.

Glufosinate Ammonium is synthetically produced as a racemate of 50 % L-isomer (active) and 50 % D-isomer (inactive) of the ammonium salts of the amino acid Phosphinothricin which has been derived from the natural compound L-phosphinothricyl-L-alanyl-L-alanine. This tripetide was obtained from *Streptomyces viridochromogenes* by Bayer et al [1] in Germany and from *Streptomyces hygroscopicus* by Kondo et al. [6] in Japan and got the common name Bialaphos. Biological screening by the agrochemical research department of Hoechst AG showed that the amino acid L-Phosphinothricin was the biologically active moiety of the tripetide. It proved to have strong herbicidal efficacy on

mono- and dicot plants when applied on leaves and other green parts. After application for patent protection [8] Hoechst developed the compound for non selective weed control uses.

Mode of herbicidal efficacy

The broad herbicidal activity of Glufosinate Ammonium is the result of a specific inhibition of glutamine synthetase [7]. In plants this is an essential enzyme for the assimilation of primary ammonia as well as for the reassimilation of ammonia released by metabolic processes. The inhibition of glutamine synthetase causes increasing concentrations of ammonia in plant cells. Finally they reach a level which is phytotoxic and destroys the plant.

Mechanism of tolerance to Glufosinate Ammonium

The *Streptomyces* species producing the tripetide Bialaphos containing the herbicidal active amino acid L-Phosphinothricin possess an enzyme - and thus a gene - which acetylates the amino group of L-Phosphinothricin. It protects the producer strain against intoxication by the own metabolite.

De Block et al. [2] proved that a Bialaphos resistance gene (named BAR gene) isolated from *Streptomyces hygroscopicus* was expressed in plants as well and protected them from herbicidal effects of Glufosinate Ammonium. Wohlleben et al. [9] isolated and characterized a Phosphinothricin resistance gene from *Streptomyces viridochromogenes*. This gene codes for an enzyme named Phosphinothricin-Acetyl-Transferase (PAT). Correspondingly the gene is called PAT gene. Although nucleotide sequences of BAR gene and PAT gene are slightly different from each other they code for similar enzymes which inactivate the active ingredient Glufosinate Ammonium, which is the common name instead of Phosphinothricin, by specific acetylation of its amino group. N-Acetyl-L-Glufosinate is formed which is no longer inhibiting glutamine synthethase and does not have any herbicidal activity.

The fact that both the herbicidal active ingredient Glufosinate Ammonium as well as the resistance mechanism have evolved in the same microorganism is an enormous advantage. They complement each other like lock and key. Adverse impacts on plant metabolism therefore are highly unlikely. This view is supported strongly by performance data of tolerant crop species in extensive field tests over several years now.

Transformation of rapeseed

After insertion of the PAT gene into *Agrobacterium tumefaciens* as transformation vector rapeseed and other dicot crops could be transformed. [11].

Using the reliable and efficient transformation technique yielded a large number of rapeseed transformants. Tolerant calli were selected on Glufosinate Ammonium containing media and regenerated to plantlets. After potting these plants were sprayed with Glufosinate Ammonium. They were completely tolerant to 2 kg a.i./ha which is about four to five times the use rate for effective weed control.

Extensive research on transformation of several different rapeseed genotypes and other plant species demonstrated that the PAT gene is stably integrated into the genome of receiver plants. As a consequence its inheritance follows clearly the rules of mendelian law. The PAT gene is furthermore inherited as a dominant gene. F1 generations which inherited the PAT gene only from one parent line, and are heterozygote in regard to the PAT gene, are as tolerant as respective homozygote generations [3].

As Glufosinate Ammonium tolerance can be easily and reliably detected, the PAT and BAR genes are used today on large scale as selection markers in transformations of many plants. They have become real tools of biotechnology and modern plant breeding.

Development of glufosinate tolerance technology

Since the PAT and BAR genes had proven to be stably integrated into rapeseed genomes the door was open to develop the Glufosinate Ammonium tolerance technology for a new dimension of selective weed control in rapeseed one of the world's important oil crops. Collaboration with major rapeseed breeding institutions and companies had been initiated in the late 1980ies. In addition own rapeseed breeding programmes have been set up in Canada and Belgium. The PAT or BAR genes are being incorporated into elite germplasm. New Glufosinate Ammonium tolerant rapeseed varieties and hybrids are in development.

The seed has to be regularly tested for Glufosinate Ammonium tolerance. The level and homogenicity of the tolerance has to be checked during all steps of breeding and seed multiplication in order to secure the highest seed quality standard possible. Only seed which meets the standard will be approved, clearly labelled and sold.

Glufosinate Ammonium is registered as a non selective herbicide in all major countries of the world. It is known for its favorable toxicological and ecotoxicological profile. Although it is registered and sold for more than 10 years its registration has to be extended to uses as a selective herbicide in genetically modified tolerant crops. Therefore data on metabolism, toxicity, residues, field performance etc. were generated for evaluation by registration authorities.

Metabolism studies with Glufosinate Ammonium tolerant rapeseed plants and other plant species confirm, that after Glufosinate Ammonium is taken up by green leaves and stems it's herbicidal active L-isomer is immediately converted to N-Acetyl-Glufosinate, the new inactived metabolite of L-Glufosinate Ammonium. The inactive D-isomer of Glufosinate Ammonium is not affecting the plant

metabolism as known from the non selective herbicide users of Glufosinate Ammonium.

The initial residue level in transgenic plants, which consists of N-Actetyl-L-Glufosinate, D-Glufosinate and lesser amounts of 3 methylphosphinico-propionic acid, decreases when the plants are growing. Residue analyses from field trials of 2 years under various growing conditions documented that at the harvest stage no residues (below limit of quantification) remain in rapeseed. Metabolism studies in soil demonstrated, that N-Acetyl-L-Glufosinate Ammonium is quickly deacetylated by microorganism to L-Glufosinate which is known already to be completely degraded to phosphate, carbon dioxide, nitrogen and water [4]. Degradation of the plant metabolite N-Acetyl-L-Glufosinate takes place the same way and as quickly as the originally applied active ingredient Glufosinate Ammonium.

Metabolism studies with rats revealed that the new metabolite N-Acetyl-L-Glufosinate is excreted very rapidly. About 90 % was found in faeces and 10 % in the urine.

Results from toxicity and ecotoxicity studies with N-Acetyl-L-Glufosinate showed no adverse effects - even at very high dose rates - as one would expect from a biologically inactive metabolite.

Safety assessments of pat gen and protein

Specificity of the PAT Protein. Incubation of purified enzyme (PAT Protein) with ^{14}C Glutamate did not result in acetylation of Glutamate. A 1000 fold higher concentration of Glutamate (or other proteingenic amino acids) could not outcompete ^{14}C labelled Glufosinate as a substrate. The extremely high substrate specificity of the PAT protein indicates that no other proteinogenic amino acid can be acetylated.

Degradation. Degradation studies of the PAT gene in digestive fluids from pork, chicken and cow showed that the DNA is completely degraded within 1 hour (at 37 °C and pH 1,5). Degradation studies of the PAT Protein in digestive fluids from pork, chicken, cow and simulated human gastric fluid resulted in an immediate breakdown of the protein and its enzymatic activity within seconds. These results demonstrate an equivalent behaviour of PAT gene and protein with other DNA and proteins of our diet.

Allergenicity. A thorough comparison of the PAT protein with other known protein sequences was conducted. No homology to known allergic or toxic proteins could be detected. All results suggest that the PAT gene and PAT protein do not pose any hazard to consumers.

Safety assessment of transgene dispersal

With the advent of transgenic crops, the danger of gene introgression from cultivated plant species into the natural flora is widely discussed. Herbicide resistance genes are in this respect an ideal approach for ecological monitoring. If the postulated gene flux from cultivated crops towards the wild flora would occur it would be easily detectable.

Rapeseed is considered as a crop which interbreeds with related wild species from the *Brassicaceae* family. Therefore in this crop the probability for outcrossing is higher than in all other agricultural crops.

Rapeseed (*Brassica napus*) arose form a cross between *B campestris* (*B. rapa*) and its *B. oleracea.* It still can be backcrossed with its ancestors. Especially crosses between *B. napus* and *B. campestris* are well documented [5], whereas outcrossings from rapeseed into *B. oleracea* under field conditions do not occur at a detectable frequency (K. Hild, personal communication). In fields in which *B. campestris* grows as a weed besides *B. napus* and the rapeseed variety confers a herbicide resistance gene, the weedy relative will be eliminated by spraying the complementary herbicide before an outcrossing can occur.

If the rapeseed field will not be treated with the complementary herbicide and outcrossing into *B. campestris* can occur, the consequence is similar to the situation of a non infested rapeseed field. Good agricultural practice will suppress and eliminate the emerging seedlings in the next crop.

Crosses of rapeseed and the two most common related weeds *Sinapis arvensis* and *Raphanus raphanistrum* do not occur under agricultural conditions even if both weeds grow in close vicinity of *B. napus.* If pollen from the wild species are brought on a stigma of *B. napus* together with *B. napus* pollen, the *B. napus* pollen grows through the pistill faster and will fertilize the egg cell. As a consequence only non hybrid seed will develop. The same is true in reciprocal crosses where the weeds are used as females. Also there the pollen of the same species fertilizes the ovaries [10]. Therefore hybrids between *Raphanus raphanistrum* or *Sinapis arvensis* and rapeseed are not detected even in rapeseed fields heavily infested with both weeds. Recently it was shown that in fields where male sterile rapeseed genotypes were grown in close vicinity of *Raphanus raphanistrum* in the absence of male fertile *B. napus* plants, a few seeds developed on the sterile rapeseed plants. Besides dihaploid rapeseed plants, intergeneric hybrids were found, amphitriploids as well as trihaploids and plants with irregular chromosome numbers [12].

The obtained interspecific F1-hybrids showed besides morphological abnormalities a reduced fertility. It is extremely unlikely that under agricultural conditions these plants can compete with well adopted fully fertile species.

These mentioned artificial conditions under which the *Raphanus-Brassica* hybrids were created do not correspond to conditions in agriculture, because a pollinator with *B. napus* pollen was completely excluded. In presence of *B. napus* pollen this would have outcompeted the pollen of the related species as described above.

In order to set the remote probability of intergeneric crosses in the right relation to agricultural reality it is helpful to remember that in a rapeseed field at harvest 3-

5 % of the seeds are falling on the ground. This corresponds to 100-150 kg seeds/ha which is equivalent to 2.000-3.000 seeds/m^2 or 20-30 mio seeds/ha.

Ever since rapeseed is grown as a crop, farmers have to handle this problem. The rapeseed seedlings which emerge from the lost seeds behave like a weed in the following crop. Appropriate cultivation practice as well as herbicide rotation solved this problem in the past as they will do in the future.

Weed resistance to Glufosinate Ammonium

Due to the mode of action of Glufosinate Ammonium it is very unlikely that weeds become resistant. The reason is that this would require a mutation of the target enzyme glutamine synthetase. However, mutated glutamine synthetase which lost its binding affinity for Glufosinate Ammonium simultaneously lost its binding affinity for glutamate, a structural analogue of Glufosinate Ammonium. A mutated enzyme therefore could not catalyze the amidation of glutamate to glutamine, the essential detoxification step for ammonia. Such a mutation would be lethal. Therefore it is extremely unlikely that weeds will develop a spontaneous resistance towards Glufosinate Ammonium.

This hypothesis is well supported:

1. Glufosinate Ammonium has been used on some areas for over 17 years several times a season. No observations of resistant weeds have been made.
2. Extensive in vitro plant selection programmes for maize and alfalfa failed to yield Glufosinate Ammonium tolerant plants. On the other hand for other herbicides it is easy to select tolerant mutants.

Field development

The first field test was done with Glufosinate tolerant spring rapeseed (Canola) in 1990 in Canada. It confirmed the excellent crop tolerance also under field conditions. In 1991 a first field test with Glufosinate tolerant winter rapeseed took place in France. In the following years the field development work was tremendously extended. Particularly in Canada the weed control trials were rapidly increased. This was possible in view of a clear regulation and approval system for field tests with genetically modified plants favoured by a close cooperation with official bodies. About 250 field trials were conducted until 1995 when the registration for selective weed control was achieved.

In Europe the regulatory and approval system turned out to be more difficult, less clear and predictable. In addition opposition against field tests with genetically modified plants was very active. For these reasons the field development work was extremely difficult to undertake and very much restricted. However, progress has been made in the last 3 years. Until now about 100 field trials with Glufosinate tolerant winter and spring rapeseed have been conducted in France, Germany and United Kingdom, which are the major markets in Europe.

Weed control trials with Glufosinate Ammonium have been conducted as post emergence applications. Single and sequential treatments with 300 to 800 g a.i./ha Glufosinate Ammonium were applied. The timing of treatments was chosen primarily according to the growth stages of weeds: Early Post, 2-4 leaves, Mid Post 4-8 leaves.

Sequential or split applications were done at Early or Mid Post followed by one further treatment in case a new flush of weeds had emerged. The spectrum of the most important weeds in spring and winter rapeseed is listed in table 1.

Table 1. Most important weeds in rapeseed

Agropyron repens	*Myosotis arvensis*
Alopecurus myosuroides	*Poa annua*
Avena fatua	*Polygonum spec.*
Capsella bursa-pastoris	*Setaria spec.*
Chenopodium album	*Sinapis arvensis*
Cirsium spec.	*Sonchus spec.*
Galium aparine	*Stellaria media*
Hordeum vulgare	*Thlaspi arvense*
Kochia scoparia	*Veronica spec.*
Lamium spec.	*Viola arvensis*
Matricaria spec.	

A lot of field trials data have been generated since the first experiment in Canada in 1990. Many of the important weeds plus a lot of less frequent ones were well controlled by dose rates of 400 g a.i./ha of Glufosinate Ammonium. Less sensitive weeds like *Viola*, *Galium*, *Cirsium*, *Hordeum* and *Agropyron* were well controlled with a higher use rate of up to 600 g a.i./ha. The higher use rate was generally necessary to control the taller weeds at Mid Post applications. In most cases a single treatment especially when applied Mid Post achieved more than 90 % overall season long weed control. According to generally more vigorous growth conditions in spring which favour the efficacy of Glufosinate Ammonium average use rates in spring rapeseed are lower (400-500 g a.i./ha) than in winter rapeseed (400-600 g a.i./ha, figure 1).

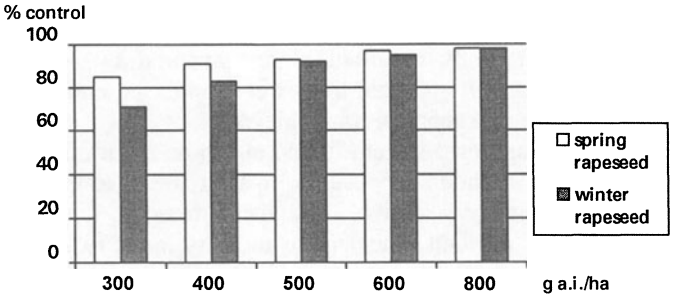

Figure 1. Average Weedcontrol with single treatment of Glufosinate Ammonium

The level of weed control with Glufosinate Ammonium was in most cases superior to standard herbicides treatments. Crop safety in Glufosinate tolerant rapeseed was excellent. Glufosinate Ammonium treated plots constantly outyielded standard herbicides treatments between 3 and 15 % or even more under Canadian Prairie conditions.

Registration and marketing

In spring 1995 AgrEvo Canada achieved worldwide the first approval for commercial use of the Glufosinate tolerant Canola variety „Innovator". Simultaneously the selective use of the herbicide Glufosinate Ammonium has been cleared and registered under the trademark Liberty. Glufosinate tolerant varieties are clearly labelled as such with the logo and trademark Liberty Link. This innovative Liberty Link weed control technology was recently also registered for corn and soybean in the United States. In Europe Glufosinate tolerant rapeseed from PGS has been cleared for seed multiplication and technical uses. Approval for food uses is expected very soon. Further registrations for Glufosinate tolerant corn and rapeseed as well as for the selective use of Glufosinate Ammonium are expected in the course of 1997.

In Canada the Liberty Link technology was launched in 1995 under a „domestic crush" program on 15.000 ha and Canola growers got excited. In 1996 they grew the Liberty Link variety Innovator and used the herbicide Liberty on about 150.000 ha with great success. A grower survey was conducted. As a result the Liberty Link Canola growers were very well satisfied. They rated the crop safety and speed of weed control of Liberty as extremely good. 84 % said they will definitely use the Liberty Link system again. The top 5 advantages of using Liberty for weed control in Canola were:

1. Broad spectrum weed control
2. One-pass weed control
3. no soil incorporation
4. firm, moist seedbed
5. soil and moisture conservation

Conclusion

There is proof that genetically modified Glufosinate herbicide tolerant rapeseed is not only a reality. The Liberty Link system has been confirmed as a new dimension of weed control in rapeseed.

The impact on weed management can be summarized as follows:

- The farmer acquires an additional option for controlling weeds after they have emerged. Nonetheless, all previous methods or products continue to be available to him.Volunteers from a previous Glufosinate Ammonium tolerant

crop can therefore be controlled by the same means as before and cause no new problems.

- Tolerance by the crop ensures maximum protection of yield. The dependence of treatment on the growth stage of the crop is reduced, thus making application easier to time. Technical management requirements can be taken into consideration to a greater degree. It is easier to keep weeds below damage thresholds.
- The new system can also make some treatments completely unnecessary (if weeds are below damage thresholds) or reduce sequential sprayings. Decisions based solely on the level of weed infestation and its development help to reduce the amount of herbicides applied.
- Glufosinate Ammonium is not incorporated in soil. Crop rotation benefits from reduced herbicide residues in the soil.
- The opportunities for soil and moisture conservation and erosion control by using different growing methods like minimum or no tillage, can be improved in combination with the new system.

Glufosinate herbicide tolerance introduced successfully as the Liberty Link system will substantially contribute to further improved cultivation of rapeseed. Better weed control combined with excellent crop safety and high use flexibility result in improved yield, quality and economics and make rapeseed a more profitable crop. Furthermore rapeseed growers are enabled to control weeds in their crop with as little side effects as possible. Consumers and the public at large will benefit from low costs of goods produced with environmentally benign methods.

References

Journals

1. E. Bayer *et al.*, *Helvetica Chimica Acta* **55**, 224-239 (1972).
2. M. De Block *et al.*, *EMBO Journal* **6**, 2.513-2.518 (1987).
3. G. Donn and P. Eckes, *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, **Sonderheft** **XIII**, 499-504 (1992).
4. E. Dorn, G. Goerlitz, R. Heusel, K. Stumpf, *Zeitschrift Pflanzenkrankheiten und Pflanzenschutz **Sonderheft** **XIII**, 459-468 (1992).
5. R.B. Jørgensen and B. Andersen, *Amer. I. Bot.* **81**, 1620-1626 (1994).
6. Y. Kondo *et al.*, *Sci. Rept of Meiji Seika Kaisha* **13**, 34–41 (1973), in: *Chemical Abstracts 1974*, **81**, 89705b
7. M. Leason, D. Cunliffe, D. Parkin, P.J. Lea, B. Miflin, *Journal of Phytochemistry* **21**, 855-857 (1982).
8. W. Rupp, M. Finke, H. Bieringer, P. Langelueddecke, *Chemical Abstracts 1978*, **88**, 70494e (1977).
9. W. Wohlleben *et al.*, *Gene* **70**, 27 (1988).

Technical reports

10. M.C. Kerlan, A.M. Chevre, F. Eber, A. Baranger, M. Renard, Risk assessment of outcrossing of transgenic rapeseed to related species: I Interspecific hybrid production under optimal conditions with emphasis on pollination and fertilization. In: *Paper of INRA* BP29 F-35650 Le Rheu France (1992).

Proceedings

11. G. Donn, R. Dirks, P. Eckes, B. Uijtewaal, Transfer and expression of modified phosphinothricin-acetyltransferase gene from *Streptomyces viridochromogenes* in tomato, melon, carrot and strawberry. In: *Abstract VII International Congress of Plant Tissue and Cell Culture Amsterdam,* 176 (1990).

Theses and unpublished material

12. A. Baranger, Evaluation en condition naturelle des risques de flux d'un transgène d'un colza (*Brassica napus*) résistant à un herbicide à une espèce adventice (Raphanus raphanistrum L.) PhD thesis 1995.

Plant Biotechnology and Implications for Rapeseed Agronomy: Development of New Methods of Pest and Disease Control

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Introduction

Today, stable food prices in developed nations is, amongst other reasons, a result of the remarkable advances in plant breeding made in the last 50 years. However, the question remains whether the lead that mankind currently has on hunger can be maintained. Before transgenic plants were an experimental reality, hope was raised that genetic engineering could be used as a tool by the plant breeder in crop improvement. Indeed, utilizing recombinant DNA technology is in essence a more precise means of carrying out what plant breeders have been doing since time immemorial. In addition to the specific manipulation of plant genes from the same as well as related or unrelated species encoding advantageous characteristics, genetic engineering provides an opportunity to transfer genes from non-plant sources into crops thus producing completely novel traits.

Plant breeders have continually incorporated new technologies that can improve the efficiency of plant breeding. Thus, biotechnology was introduced into conventional breeding programs about 15 years ago. Initial goals of biotechnological plant breeding was to improve crop varieties for food and feed use by engineered resistance against biotic and abiotic stress, resistance against herbicides as well as stabilization and increase of yield. Genetic engineering (molecular breeding) is aimed at developing crops with improved characteristics for an ecologically sustainable agriculture, either for food and feed or for industrial use.

Plant protection against pests became a major challenge to agriculture worldwide to maintain stable food prices and continuous food supply for growing populations. Significant yield losses are caused in any crop by bacterial, viral, fungal, nematode or insect pests which can reach in developing nations routinely 40-50 % or even higher (table 1).

Table 1. Importance of Plant Protection

Average Yield Loss world wide ca. 25 %

Average Yield Loss in Developed Countries	± 15 % (under Plant Protection)
Average Yield Loss in Developing Nations	- Rice ± 45 %
	- Sugar Cane ± 55 %
	- Corn ± 35 %
	- Millet ± 35 %
	- Cotton ± 35 %

Average Yield Losses under a „Ban of Agrochemicals"

Crop	Ban of Plant Protection
-Potato	± 45 %
-Oilseed Rape	± 40 %
-Winter Wheat/Barley	± 35 %
-Sugar Beet	± 37 %

On a worldwide basis yield losses reach around 25 %. Presently, control of pests and diseases is mainly achieved by application of chemicals. Still pathogens cause major losses of 10-15 % in an agriculture with chemical protection. Taken together, a fair number of strategies already exist to control plant pests by molecular breeding. The use of genetic engineering in resistance breeding, being an essential part of integrated pest management in combination with chemical control, traditional breeding and crop rotation, clearly could lead to a more ecologically sustainable agriculture (table 2).

Table 2. Plant Protection - Integrated Pest Management

Combined Strategy towards a more sustainable Development of Modern Agriculture consists of

Pesticides
- Fungicide, Insecticide, Herbicide

Physical Measures
- Heat, Mechanical Treatment

Biological Measures
- Phermones, Bt-Technology, beneficial Insects, biological Weed Control

Farming Measures
- Soil, Fertilizer, New Varieties

including as Integrative Part of Pest Management

Biotechnology

Resistance to fungal pathogens

Fungal diseases have been among the principal causes of crop losses ever since humans started to cultivate plants. To date the epidemic spread of fungal diseases is controlled by various crop husbandry techniques, breeding of resistant cultivars and the application of agrochemicals (fungicides). Although breeders have succeeded in producing cultivars resistant to fungal diseases, limitations in the availability of natural resistance genes and time consuming breeding processes make it difficult to react adequately to the evolution of new virulent fungal races. This is especially the case with rapeseed, corn, potato and wheat where either fungal pathogens rapidly break the resistance breed or simply no breeding leads are available yet. Although much energy and efforts have been invested into biotechnology of fungicidal plants, the technology is somewhat behind the commercial success of e.g. *B.t.*-insecticidal plants. Proven principles to engineer plants resistant to fungal diseases do exist and have already been tested in field trials. However, research and marketing efforts are just being intensified as a consequence of positive experimental data during the last 3 years or so. Currently much energy is being applied to identifying and isolating antifungal genes of all sources available, including microorganisms. Some of these efforts are being focused on resistance genes known from conventional breeding programs. Furthermore, there is an extensive search for genes that encode enzymes involved in the synthesis of compounds toxic to fungi and for genes that encode proteins with a direct inhibitory effect on the growth of fungi. Moreover possibilities to exploit genes encoding inhibitors for fungal enzymes (pectolyase, polygalacturonidase, cellulase) to obtain resistance are being investigated.

Table 3. Strategies for Engineered Resistance to Fungal Pathogens

1: Improved Resistance via Antifungal Proteins
- chitinases and glucanases (plant, microbial)
- ribosome-inactivating proteins (RIPs)
- pathogenesis related (PR) proteins (e.g. PR 5)

2: Improved Resistance via Multigene Defense Mechanisms
- enhancing stress metabolism (anionic peroxidase/ glucose oxidase)
- modifying phytoalexins (e.g. stilbene synthase)

3: Manipulation of Regulatory Mechanisms
- artificial local cell death (HR; BARNASE/BARSTAR system)
- isolation and use of natural resistance genes (avr-/R-gene system)

Improved resistance via antifungal proteins: During the last years, some genes involved in plant defense have been successfully used to enhance resistance to various fungal pathogens in transgenic plants (table 3). Several characteristic features of fungal walls e.g. chitin and glucans, as well as extracellular growth of several pathogenic fungi are potential targets for inhibition of fungal growth in genetically transformed plants. Antifungal proteins, such as ß-1,3-endoglucanases and chitinases, aimed towards these targets, or ribosome inactivating proteins (RIPs) have been used as defense transgenes. Recently it became clear that

constitutive expression of a bean chitinase in *Nicotiana tabacum* as well as in *Brassica napus* transgenic plants resulted in enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Meanwhile, other studies demonstrated that ectopic expression of antifungal proteins, also including an antifungal protein from the mold *Aspergillus giganteus* (AgrEvo GmbH), in transgenic plants, can lead to enhanced protection against different fungal pathogens such as *Alternaria alternata, Cercospora nicotianae, Fusarium oxysporum, Peronospora tabacina, Phytophthora parasitica* and *Rhizoctonia solani*. As plants naturally respond to fungal attack by expression a whole set of defense genes it remained to be seen whether combined expression of antifungal genes is also of use for strategies of genetically engineered resistance. It became clear that overexpression of chitinase and glucanase simultaneously in transgenic plants could lead to drastically enhanced resistance to fungal pathogens by synergistic interaction of the antifungal proteins, which is not limited to the combination of different hydrolytic enzymes. Likewise, breeding for fungal resistance in transgenic plants with „multi gene" combinations may make good sense since it is likely that this „multi gene" resistance strategy could result in a reduction of the probability of the emergence of resistance breaking strains of pythopathogenic fungi.

Most important symbiotic fungi can also colonize plant tissues without causing any damage to their host and are often beneficial to their growth. Although plants naturally respond to pathogenic fungi by a complex network of defense reactions, symbiotic fungi have found a way to avoid or overcome the defense mechanisms of the host plants. Genetically engineered resistance to fungal diseases is therefore only of value to agriculture if beneficial symbiotic fungi are not affected. Right now it appears possible that genetic engineering makes use of the antifungal properties of PR proteins in transgenic plants without reducing their symbiotic potential. However, it would be hasty to draw general conclusions. More pathogen resistant transgenic plants expressing various defense genes will have to be evaluated for their symbiotic ability.

Improved Resistance via multigene defense mechanisms: Other strategies are currently being tested which would provide further tools to enhance resistance to fungal diseases by pyramiding genes in form of different combinations in one and the same plant. Molecular tools to enhance stress metabolism, e.g. by expression of anionic peroxidase or glucose oxidase, are able to trigger natural plant signals for defense responses (table 3). Also plant secondary metabolites, which are part of the natural plant defense system to fight fungal diseases, can be modified. Expression of a heterologous stilbene synthase in tobacco has led to accumulation of the phytoalexin resveratrol, which effectively protected transgenic plants from *Botrytis cinerea* attack. Other genes coding for enzymes which control plant secondary metabolites could be of similar use to create fungicidal plants (table 3).

Manipulation of regulatory mechanisms: Last but not least, isolation of natural resistance genes and fungus-responsive promoters during the last few years opened an exiting way to create an artificial hypersensitive system, which is quite common as a natural defense response in plants. Here, the localized cell death in response to fungal infection, which consequently limits further spread of the fungus, is engineered (HR; BARNASE/BARSTAR system; natural defense

response; avr-/R-gene system, table 3). It remains to be seen how these transgenic plants behave under natural field conditions.

The first examples of genetic engineering of resistance to fungal diseases clearly demonstrate the potential of this technology, although such crops have not reached the market yet as in the case of insect resistance. A combination of different antifungal principles would further enhance the protective properties of the plants and probably reduce the emergence of resistance breaking strains. Although first principles do exist, the next years will dramatically increase the knowledge of how to engineer plants resistant to fungal diseases. Undoubtedly, in 5-10 years from now, engineered fungicidal crop plants will play a significant role in pest management systems.

Resistance to plant-feeding insects

Plant biotechnology has become a source of agricultural innovation, providing new solutions to old problems. The costs associated with management and chemical control due to insect pests still reach 10 billion $ annually, Major losses of 20-35 % of the total production still occur. In view of this genetic engineering represents an attractive opportunity to reduce insect damage with clear advantages over traditional chemical control. Over the past few years the success in generating insect-resistant crops by genetic engineering has been impressive. Consequently this has led in 1996 to the first generation of plants expressing insecticidal proteins introduced into the marketplace in U.S.. The next generation of insecticidal plants will use novel principles and combinations to control important insect pests, which have escaped from the first strike of insect control biotechnology.

Table 4. Strategies for Engineered Resistance to Insect Pests

1: *Bacillus thuringiensis* δ-Endotoxins Expressed in Plants
   - expression of truncated *B.t.*-proteins and synthetic *B.t.*-genes
   - large diversity of *B.t.-toxins* against different pests
2: Expression of Protease Inhibitors
   - cowpea trypsin inhibitor (CpTI)
   - several others available
3: Engineered Expression of Insect Specific Peptide Hormones
   - e.g. Trypsin Modulating Oostatic Factors (TMOFs, 6-10 aa)
   - allatostatin (5 aa) or proctolin
4: Manipulation of Plant Secondary Metabolites
   - phytoecdysteroids

Technology used today. The concept of creating insect resistant plants began with the identification of insecticidal proteins. The soil microorganism *Bacillus thuringiensis* (*B.t.*) has proven to be an extremely rich source of insecticidal proteins and genes. The *B.t.*-δ-endotoxins exert their toxicity by binding to the midgut epithelian cells and ultimately causing osmotic lysis through pore

formation in the cell membrane. Known *B.t.*-strains contain great δ-endotoxin diversity and specificity to insects and over 300 different toxins have been reported right now, the number of which still is increasing. Efforts to enhance *B.t.*-gene expression in plants became a priority and tremendous success has been made by optimizing the codon usage, thus leading to completely synthetic *B.t.*-genes. By doing so for example the expression in transgenic plants could be increased nearly 100-fold. This has led to transgenic plants with strong resistance to the respective insect pests (cotton-*cry1Ab/cry1Ac-Heliothis zea*; potato-*cry3A-Leptinotarsa decemlineata*; corn-*cry1Ab-Ostrinia nubilalis*, table 4).

Future technology. Although most actual insecticidal transgenic plants have been based on *B.t.*-toxins, many research projects were started to discover non-B.t. proteins and principles to control insect pests. For instance, polyphenol oxidase generates toxic compounds from dietary components. Proteinase and amylase inhibitors deprive the insect of nutrients by interfering with digestive enzymes of the insects. The insecticidal properties of chitinase have also been explored, which targets chitin structures such as the midgut peritrophic membrane. Lectins constitute another large family of proteins with certain insecticidal properties and it remains to be seen how transgenic plants perform under field conditions. Surprisingly cholesterol oxidase turned out to have insecticidal effects when added to the diet. Physiologists suggest that cholesterol oxidase modifies cholesterol within the cell membrane, which consequently destroys integrity and normal function of the exposed cell membranes in the insect digestive tract (table 4).

Insect hormones. For quite a long time it was believed that insects do not have hormones. Today we have evidence that the endocrine system of insects is as complex as that of vertebrates. The two classical non-peptide hormones belong to the class of juvenile hormones and ecdysteroids, the moulting hormone. Some plants contain huge amounts of ecdysteroids of which the 20-OH-ecdysone is amongst the most potent insecticides known. The chemical structure is too complicated to allow easy and cheap synthesis or controlled genetic manipulation of the ecdysteroid secondary pathway in plants. However, the recent discovery of the biosynthetic pathway of brassinosteroids and isolation of the genes made it worth while to speculate whether in future new generations of transgenic plants could be foreseen, which make use of the ecdysteroid hormones for insect population control. A large number of peptides also exist which control the endocrine system of insects. The field exploded from 1985 on and today approximately 100 insect peptides have been discovered. The list of insect peptide hormones still continues to grow. All these insect regulatory peptides principally have the potential to control insect populations. Actual research is therefore aimed to find out which peptide is still active after oral uptake and expression in transgenic plants. First positive data in transgenic plants with e.g. proctolin exist (table 4).

Further future technologies. Strategies emerge to create new generations of insecticidal plants. A combination of different insecticidal strategies, including combined expression of different *B.t.*-toxins in one and the same plant, could further enhance protection against insect pests and reduce the probability of

emergence of resistance breaking insect populations. It can be expected that in about a decade from now, the only proteinaceous toxin (*B.t.*-toxin), which is now routinely used for insect control, will have neuropeptic companions of insect origin in transgenic insecticidal plants. Although the evaluation of the insect hormone system as a target to engineer plants resistant will take some time, enormous potential to create insecticidal plants exists hand in hand with approaches such as *B.t.*-toxins, protease inhibitors or enzymes such as cholesterol oxidase (table 4).

Strategies for engineered resistance to plant viruses

Coat protein mediated resistance: Plant viruses are difficult to control as no „viricides" can be developed. Conventionally, virus spread can only be controlled by farming measures, certified virus free planting material and chemical control of virus vectors such as aphids. In addition, engineering of virus resistance is expected to provide a more direct and efficient control of diseases. Basically, engineering of virus resistance is based on the introduction of virus-derived genomic sequences or virus-targeted sequences. The first approach, which has been tested in 1986, was the coat protein mediated resistance. The group of R. Beachy introduced the Tobacco Mosaic Virus (TMV) coat protein gene into tobacco plants. Efficient protection against TMV infection was observed via this protein also under field conditions. In the following years the coat protein of several plant viruses (viruses of rice, potato, wheat or tomato plants) have been used successfully to generate resistant transgenic plants expressing these coat proteins.

Replicase/movement protein mediated protection: Another approach followed is to express defective virus proteins in the plants, which interfere with normally active viral proteins simply by competition for the respective binding sites or functions. A defective replicase produced by transgenic plants was able to compete with viral protein for the binding sites on the viral genome, thus preventing viral replication and development of disease. In a similar way a defective movement protein expressed in transgenic plants was able to block cell to cell spread of the virus.

Antisense strategy/ co-suppression: Other strategies make use of small, catalytically active RNAs (Ribozymes) expressed in transgenic plants. They specifically cleave the viral genome in case of infection. Expression of virus-genomic sequences in antisense orientation also led to effective control of virus disease. Here, the antisense RNA in the cells hybridizes with the corresponding viral sense RNA and prevents it from being translated. This already has led to transgenic plants resistant against to a whole spectrum of viruses. However, surprising was the fact, that untranslatable viral RNA or parts of the viral genome, when expressed in sense orientation in transgenic plants, were able to control viral diseases. This phenomenon has been called co-suppression and was effectively used in the last years to engineer plant virus resistance. Still the molecular

mechanism for co-suppression is unclear. The favoured hypothesis is that plants are able to monitor excess of a distinct RNA and turn on an effective control mechanism to reduce viral RNA during the infection process

Principles to obtain plants resistant to bacteria and nematodes

Bacterial diseases are of economic importance in many crop plant species including potato, different vegetable species, fruit trees, rice, cotton and grapevine. Resistance traits known to be present in species often cannot be used for classical breeding as the plants are genetically too distant from the cultivated species. Bacterial diseases are therefore mainly controlled by phytosanitary practices and highly developed harvesting techniques to minimize wounding. Chemical control is difficult to achieve as no „bacteriocides" for large scale field use exist. Genetic engineering now is hoped to control bacterial infection mainly by expression of bacteriostatic proteins.

Lysozyme: For a long time the expression of lysozyme has been optimized as lysozymes specifically degrade bacterial cell wall components. This has led to some success in transgenic potato against *Erwinia carotovora* infection. Genes for antibacterial proteins from plants (thionins), insects or frogs (cecropin, maganin) also have the potential to engineer plants resistant bacterial diseases. Surprisingly, the expression of glucose oxidase gave some resistance to *E carotovora* in potato. Other approaches, such as antibody-based specific inhibitors of pathogenic factors will be tested in future for their potential. The approaches outlined provide an encouraging potential for genetic engineering to solve some problems of susceptibility to bacterial diseases in crops where conventional breeding has no lead yet.

Hypersensitive cell death (HR-response): Many species of plant parasitic-nematodes have evolved complex relationships with their host, inducing profound alterations in the morphology of the cells from which they feed. As parasites they mainly compete with the plants for nutrients and reduce uptake of water when heavily damaging the root system. This consequently leads to reduced and stunted plant growth. A strong stimulus for designing novel strategies for the engineering of nematode resistance by genetic engineering were the limitations on the use of nematocides. Increased environmental concern and relatively high toxicity has led to a ban or reduced use of a lot of nematocides in various countries. As a consequence approaches to genetically engineer plants resistant to nematode infection emerged rapidly. A number of potentially effective anti-nematode genes have been reported. These reports include expression of collagenase with the aim to damage specifically the animal's cuticle. Although this approach is not fully developed yet, it has high potential in the future in case the collagenase shows specific activity against nematodes.

Other strategies: Furthermore, nematicidal *B.t.*- strains have been isolated and it remains to be seen whether the *B.t.*-toxins can confer resistance to the transgenic

plants against nematode attack. Antifeedants such as the cowpea trypsin inhibitor (CpTI) might also have potential against nematodes. Specific expression of cytotoxic compounds can be used in this defense strategy as well as a form of artificially engineered cell death, which specifically could led to a collapse of the feeding cells of nematodes in the roots. A fair number of nematode resistance strategies actually do exist which are under evaluation with transgenic plants in field trials. The potential does exist and will be clear soon. However, a major breakthrough represents the recent report of Cai and colleagues [1]. They cloned the first natural nematode resistance gene against the cyst nematode *Heterodera schachtii* from sugar beet. Molecular analysis revealed that this probably is a receptor, which opens exiting ways for strategies to use natural plant defense systems against nematodes in genetic engineering.

Rapeseed as target crop

Rapeseed varieties are cultivated worldwide in moderate climatic regions and mainly grown for their seed. The seed of *Brassica* spp. typically contains about 40 % oil and the remaining seed meal after oil extraction has high protein content, which is suitable for animal feed. Yields vary around 1-1.8 t/ha oil depending on local agricultural conditions. Typical cultivated Brassica species are *B. napus* (Europe/Canada/China - summer rapeseed (SOSR) and winter rapeseed (WOSR)), *B. rapa* (Canada/India - SOSR/WOSR), *B. juncea* (India/China/Australia), *B. carinata* (Ethiopian plateau/East Africa) *and Sinapis alba* (Europe/ N. America). However, species grown preferentially are *Brassica napus* and *Brassica rapa*. The areas cultivated with *B. napus* and *B. rapa* species are mainly restricted to Canada (5.0 Mio ha), China (5.0 Mio ha), India (4.5 Mio ha) and Northern Europe (4.0 Mio ha).

Yield losses in rapeseed can be quite high due to pests. Weeds, insects and fungal diseases contribute equally to yield losses up to 25 % under the regime of plant protection. To control weeds new strategies by use of genetic engineering have been developed which make use of engineered plants resistant to the herbicide glufosinate (BASTA®). Bacteria, nematodes and viral pathogens (e.g. *X. campestris, E. carotovora, H. schachtii,* CMV, CaMV, BWYV) do not cause major problems in cultivation of rapeseed and are only of local importance. Application of chemicals against the pathogens or vectors and cultivation methods easily control the diseases.

However, fungal pathogens and insect pests are of major importance for rapeseed cultivation and breeding therefore is aimed to genetically engineer resistance to the major fungal and insect pathogens. A lot of strategies do already exist to develop resistance to fungal diseases and first *in vitro* assays revealed that the antifungal principles also work against phytopathogenic fungi such as *Phoma lingam* and *Sclerotinia sclerotiorum* (others: *A. brassicae, V. dahliae, P. parasitica*). Especially engineered resistance against *Sclerotinia sclerotiorum* would be a major breakthrough because breeders actually have no lead to control the disease.

Insects pests are of major importance for rapeseed cultivation as well, because they also support fungal attack by damaging the leaf or plant organs. Main insect pests worldwide are *Plutella xylostella* (Diamondback moth), *Mamestra configurata* (Cabbage moth), but also *Meligethes aeneus* (Pollen Beetle), *Psylloides chrysocephala* (cabbage stem flee beetle) and *Ceutorhynchus* spp. (Stem weevils) can cause great local problems. Here, specifically active *B.t.*-toxins to transfer the genes into plants would be a major challenge. First data do emerge that some *B.t.*-toxins do exist, which are active against *Plutella xylostella* and *Mamestra configurata*. The respective genes could be used to transform rapeseed varieties, thus engineering resistance to this insect pests.

Conclusions

Transgenic plants are now entering the marketplace. Currently, tomatoes with enhanced storage life as well as puree are commercially available in the U.S. and the U.K., respectively. Cotton and corn plants engineered to be resistant to insect predation. Herbicide tolerant rape, corn and soybean have been approved for commercialization in Europe, U.S. and Canada. These examples are a small fraction of the potential of transgenic plants. The creation of plants resistant to microbial pathogens, insects and probably nematodes is also feasible. Furthermore resistance to environmental stress, along with modified growth, or biochemical characteristics might also improve plant quality. Indeed, it can be envisaged that in the future transgenic plants will play a significant part in modern agriculture. These advances, coupled with a better knowledge of the structure of the plant genome is likely to revolutionize plant breeding in future.

The last years several strategies are becoming available for molecular breeding to improve resistance of transgenic plants against pests. Generally, transgenic plants expressing antifungal proteins (chitinase, glucanase and RIP) have been effectively protected against a variety of fungal diseases, whereas symbiotic mycorrhizal fungi remain unaffected. Other antifungal strategies, such as artificial localized cell death, do exist for pyramiding strategies against fungal diseases. Insect predation has been controlled by expression of insect specific proteins from the bacterium *Bacillus thuringensis* (*B.t.*-toxin). A combination with other genes coding for insecticidal proteins in a transgenic plant could further enhance protection of plants against insect pests. Control of viral diseases in transgenic plants was achieved by overexpression of coat- or movement protein from the virus itself, which limits replication and spread in the plants. Other viral genes, or subgenomic fragments, either in sense or antisense orientation effectively conferred resistance to viral diseases. Several strategies also become available to engineer resistance against bacterial diseases and nematode attack. Expression of proteinase inhibitors, active against nematodes, or specific physiological manipulation which leads to the collapse of feeding cells of sedentary nematodes has been shown to control nematode pests. This demonstrates that a fair number of strategies already exists to control plant pests by molecular breeding. In several cases a combination of different resistance strategies in one and the same plant has

been shown to exert synergistic protective effects. In future, this probably will reduce the emergence of resistance breaking strains leading to genetically engineered plants with improved and stable resistance characteristics. The use of genetic engineering in resistance breeding as part of integrated pest management clearly could lead to a more ecologically sustainable agriculture.

Relevant Literature

1. D. Cai, M. Kleine, S. Kifle, H.-J. Harloff, N.N. Sandal, K.A. Marcker, R.M. Klein-Lankhorst, E.M.J. Salentijn, W. Lange, W.J. Stiekema, U. Wyss, F.M.W. Grundler, C. Jung, *Science* **275**, 832 (1997).
2. B.J.C. Cornelissen and L.S. Melchers, *Plant Physiol,* **101**, 709 (1993).
3. K. Düring, *Mol. Breeding* **2**, 297 (1996).
4. J.J. Estruch, N.B. Carozzi, N. Desai, N.B. Duck, G.W. Warren, M.G. Koziel, *NATURE/BIOTECH* **15**, 137 (1997).
5. S.S. Gill, E.A. Cowles, P.V. Pietrantonio, *Ann. Rev. Etomol.* **37**, 615 (1992).
6. J.S. Feitelson, J. Payne, L. Kim, *BIO/TECH.* **10** (1992).
7. G. Jach, B. Görnhardt, J. Mundy, J. Logemann, E. Pinsdorf, R. Leah, J. Schell, C. Maas, *Plant Journal* **8**, 97 (1995).
8. J.D.G. Jones, *Nature* **385**, 397 (1997).

A. D. Loof, *Agro-Food-Industrie Hi-Tech, Nov/Dec.* **23** (1996).

9. P. Nicolas and A. Mor, *Ann. Rev. Microbiol.* **49**, 277(1995).
10. P.C. Sijmons, H.J. Atkinson, U. Wyss, *Ann. Rev. Phytopathol.* **32**, 235 (1994).
11. G. Strittmatter and D. Wegener, *Z. Naturforschung* **48**, 673 (1993).
12. R. Walden, C. Maas, N. Martini, J. Schell, *Europ. Review* **4**, 393 (1996).

Modification of Fatty Acids by Genetic Engineering

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The oils derived from temperate oilseeds have a fairly narrow range of fatty acid distribution. With the exception of the crucifers, most have oils that predominantly consist of C18-fatty acids with varying degrees of desaturation. Although common in tropical oilseeds and many wild species, fatty acids with shorter than 16-carbon chain length are virtually absent from temperate oilseeds.

Genetic engineering has made it possible to transfer genes between widely different taxa and thus has facilitated modifications of fatty acids to much higher degree than what was possible via mutagenesis or interspecific crosses between close relatives. *Brassica napus*, a species of rapeseed has been a very successful target for genetic engineering. The same species has also been successfully used in modifications of fatty acid profiles by means of conventional plant breeding, resulting in types such as high erucic rapeseed (closest to wild type), low erucic rape, low linolenic rape and high oleic rape.

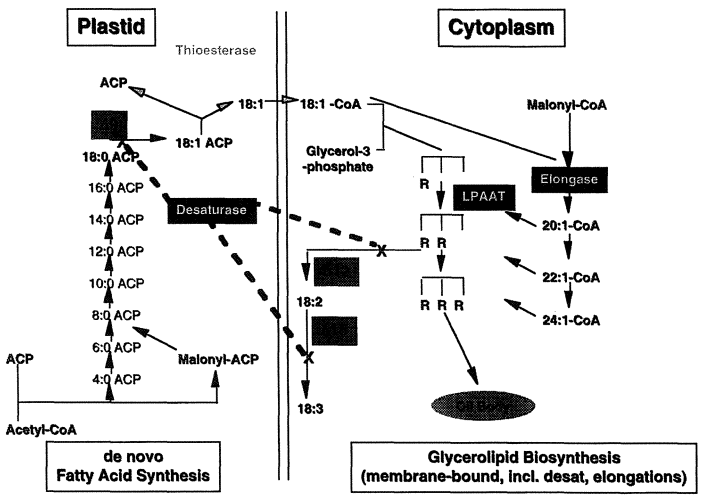

Figure 1. The biochemical pathway

The synthesis of plant oils involves a number of steps that can be targeted for modification by genetic engineering (figure 1). In plastids keto-acyl ACP synthase (KAS) enzymes facilitate the elongation of fatty acids in ACP-thioesters by adding to the chain two carbons at a time. The elongation process is terminated by thioesterase (TE) enzymes that hydrolyze the fatty acid chain from ACP once it reaches the length targeted by the specific TE (typically 18 carbons in temperate oilseeds). In plastids, stearoyl-ACP may also undergo desaturation facilitated by delta-9 desaturase (D9D), leading to monounsaturated oleic acid to be released to the cytoplasm compartment. In the cytoplasm, the fatty acids may be desaturated further by other desaturases. Other enzymes can attach components like hydroxyl and epoxy groups into the fatty acid. Fatty acid elongase (FAE) enzymes may also add additional carbon groups to a 18-carbon fatty acid, resulting in synthesis of very long chain fatty acids (VLCFA). Finally, different acyltransferase enzymes esterify specific fatty acids to each of the three positions in the triglyceride molecule. The specificity of these enzymes also affects the characteristics of the oil and sets certain limits to the contents of different fatty acids.

Modification of the fatty acid composition of rapeseed oil was started at Calgene 1985. Several critical assumtions had to be made about the fatty acid biosynthesis and the possibilities to modify it without adversely effecting the viability of the plant (table 1).

Table 1. Initial risk factors

- All plants sythesize seed oils using the same basic biosynthetic pathway
- Oil enzymes from one plant species would work in the pathway of another plant species
- Pathway is well enough characterized to allow praticable modification
- Key enzymes can be purified and genes cloned in a reasonable time-frame with reasonable resources
- Possible to make signifcant changes in the oil without adversely affecting plant viability

One of the first goals for this work was development of rapeseed oil containing high concentrations of lauric acid (C12:0). Several differnt sourcess for laurate specific thioesterases were studied, some of which are listed in table 2.

Table 2. Potantial sources of laurate thioesterases

Plant	C8:0	C10:0	C12:0	C14:0	C16:0	C18:1
Cuphea						
- mimuloides	-	-	25.0	10.0	20.0	10.0
- graciliflora	-	4.8	78.0	9.6	3.0	2.9
- setosa	-	1.1	62.2	26.0	5.3	1.3
Palm Kernel	3.5	3.5	48.0	16.o	8.0	15.5
Coconut	8.0	6.0	47.0	17.5	9.0	7.0
Bay Laurel	0.5	28.1	66.7	1.6	0.2	1.9

Finally, introduction of a TE from the California bay tree, *Umbellularia Californica* to low erucic *B. napus* resulted in a type of rapeseed containing up to 50 % (weight) lauric acid in the oil. This type is now in commercial production in North America and the oil is finding wide acceptance in both edible and non-edible applications. The typical composition of commercially produced laurate canola oil is given in table 3.

Table 3. FA composition of laurate canola

Fatty Acid	Laurate Canola	Commodity Canola
C12:0	40.0	-
C14:0	4.0	0.1
C16:0	0.3	4.0
C18:0	1.5	1.5
C18:1	29.4	61.5
C18:2	10.8	20.0
C18:3	8.6	10.0
Other	5.4	2.9

Introduction of a TE gene from *Cuphea hookeriana* resulted in rapeseed that contains significant amounts of medium chain fatty acids C8:0 and C10:0 (figure 2). The researchers at Calgene were also able to enhance the content of C8:0 and C10:0 and adjust the balance between them by introducing a gene coding for a KAS enzyme from *C. hookeriana*. Engineering TEs from other *Cuphea* species and nutmeg into rapeseed has also resulted in oils with high percentages of C14:0 and C16:0 (figure 3).

Development of high stearic and high oleic rapeseed oils via genetic engineering was made possible by the work done at Calgene and by our colleagues at DuPont. The very first genetically engineered oil was a high-stearate canola produced by shutting off the gene for D9D using Calgene's antisense technology. This involves inserting the genes into plants in reversed orientation which causes the expression of the endogenous gene to be blocked or reduced. Another mechanism called co-suppression was used by the group from DuPont to produce a high oleic oil by turning off the desaturases that convert oleic acid to C18:2 and C18:3. Co-suppression involves expressing genes in plants at such high levels it causes the plant to turn off the endogenous gene. Further improvement in stearic acid content was achieved at Calgene by introducing a TE-gene from mangosteen to rapeseed.

Increases in longer chain fatty acids was achieved by inserting to *B. napus* keto-acyl CoA synthase (KCS) enzymes isolated from jojoba and *Lunaria annua*. The effect of the jojoba KCS appears to be analogous to Brassica KCS, compensating for the dysfunctional C22:1 elongase gene in low erucic rapeseed (figure 4), while the expression of the *Lunaria* KCS resulted in greatly increased content of nervonic acid, C24:1 in high erucic rapeseed (figure 5).

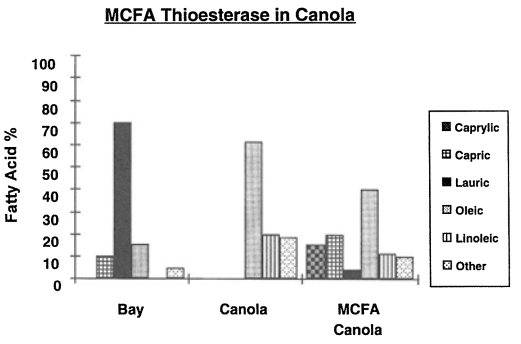

Figure 2. Medium chain fatty acids

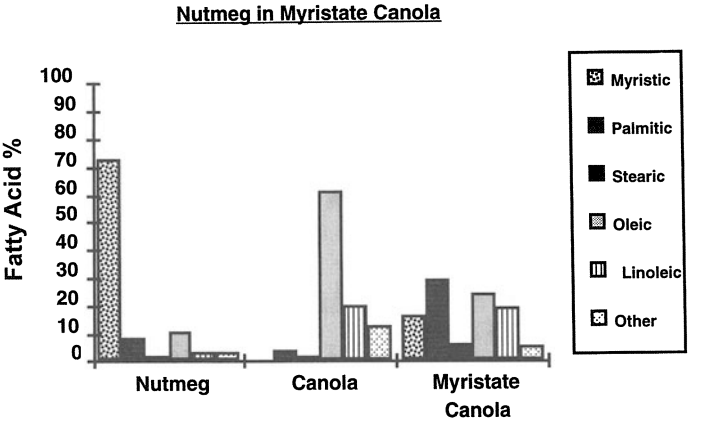

Figure 3. C14:0 fatty acids

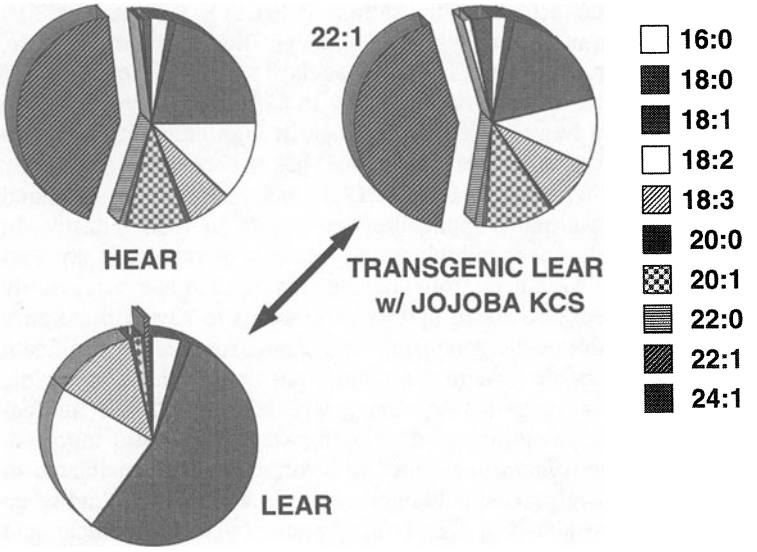

Figure 4. Production of HEAR using jojoba KCS enzyme

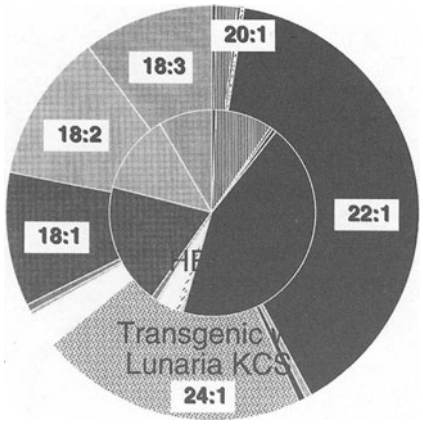

Figure 5: HEAR vs. transgenics with Lunaria

Rapeseed lysophosphatic acid acyl transferase (LPAAT), the enzyme that facilitates the esterification of fatty acid in the middle position in the triglyceride, has a strong preference for unsaturated C18-fatty acids. This limits the content of fatty acids with different chain length theoretically to 67 % and in practice even this level has turned out to be very difficult to reach in high erucic rapeseed. In products such as laurate canola this preference has a beneficial effect by increasing the content of type C12:0-C18:n-C12:0, and results in a structured triglyceride product that has many applications especially in food industry. In oleochemical applications however, a higher concentration of required fatty acid would be beneficial. An LPAAT gene from coconut was isolated and successfully inserted to rapeseed. The enzyme coded by this gene seems to have a preference for C12:0 and the expression of the gene in laurate canola resulted in significant increase in the frequency lauric acid in the middle position of the triglyceride. Progenies from crosses between plants expressing high levels of bay TE and coconut LPAAT indicate the possibility of developing very high lauric rapeseed. Similarly, LPAAT gene from *Lunaria* resulted in incorporation of erucic acid in the middle position of the triglyceride in high erucic rapeseed (figure 6) and when combined with enhanced synthesis of C22:1 may result in very high erucic acid rapeseed.

It now seems that most if not all aspects of fatty acid and triglyceride biosynthesis can be modified by means of genetic engineering, which will in future lead to development of vegetable oil products with greatly improved utility in different edible and non edible applications. Some commercial specialty oils as well as those currently under development by Calgene are given in table 4.

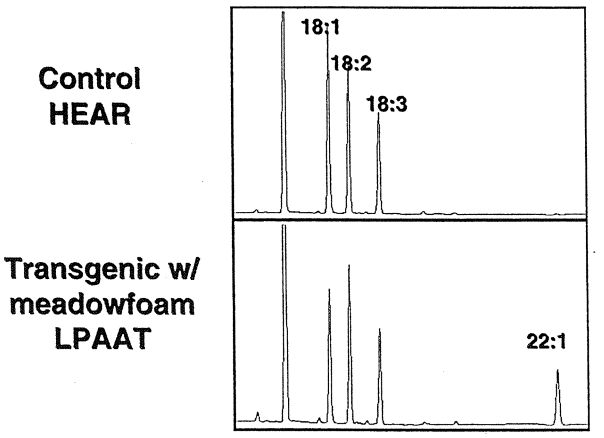

Figure 6. sn-2 composition of HEAR oil

Table 4. Oils product matrix

Project	Market Food	Nutritional	Lubricants	Oleochemical	Cosmetic
Laurate	x		x	x	x
Myristic	x			x	x
MCFA/MCT	x	x	x	x	
Stearate	x				
Palmitate	x				

Genetic engineering of vegetable oils will impact Biodiesel markets both directly and indirectly. The chain length and saturation level will have an impact on the functionality of the oil as raw material for diesel fuel. Genetically modified oils with unique chemical composition will provide users with more desireable and efficient raw materials thereby impacting the functionality of Biodiesel. Genetic engineering will also result in a number of specialty fatty acids and other ingredients being produced in rapeseed oil (table 5). The production of such specialty value-added ingredients will increase the supply of available basestocks and by-products for conversion to fatty acids and methyl esters. Improving the economics of Biodiesel production may in the final analysis be at least as important as improving functionality and genetic engineering can play an important role in both areas.

Table 5. Future products based on genetic engineering

- PUFA
  - Gamma Linolenic acid (GLA)
  - Docosahexanoic acid (DHA)
  - Eicosapentanoic acid (EPA)
  - Arachidonic acid (ARA)
- Carotenoides
- Enzymes

Breeding of High-Oleic-Acid Rapeseed (HOAR)

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The breeding programme of high-oleic-acid rapeseed (HOAR) was started at NPZ/Lembke almost 10 years ago. The idea was to improve the quality of rapeseed oil for human consumption as well as for oleo-chemical applications. Recently we have learnt that this new quality will also improve the Biodiesel fuel for diesel engines because this new rapeseed oil will have a lower iodine number and, therefore, result in a better RME-product.

The biochemical pathway of fatty acid biosynthesis is well known in rapeseed (figure 1). Elimination of erucic acid (C22:1) was the first step to increase the oleic acid (C18:1) level from about 15 % to 60 % (table 1). All the presently grown double low (00-) rapeseed varieties contain about 60 % oleic acid in their seed oil.

Table 1. Fatty acid composition from different rapeseed genotypes

	Percent fatty acid of total lipids						
Type	16:0	18:0	18:1	18:2	18:3	20:1	22:1
High erucic	4,5	1,5	15,0	13,0	10,0	6,0	50,0
Low erucic	4,5	1,2	60,0	22,0	12,0	0,1	0,2

For starting the breeding programme for HOAR the following genetic variation was used:

1. The natural, genetic variation of oleic acid content within double low breeding materials. In our screening programmes variation between 54 % to 65 % was found with some extreme values of up to 70 % oleic acid content. Very often high contents represent just phenotypic and not stable genotypic values. Nevertheless, fixed lines with about 68 % to 70 % oleic acid content could be selected within our 00-breeding materials.
2. Chemically induced, low linolenic mutants. From earlier experiments [1,2] mutants were available with less than 2 % to 3 % linolenic acid (C18:3). This biosynthetic block should contribute indirectly to the oleic acid content, although most of these mutants have also an increased linoleic acid content (C18:2).

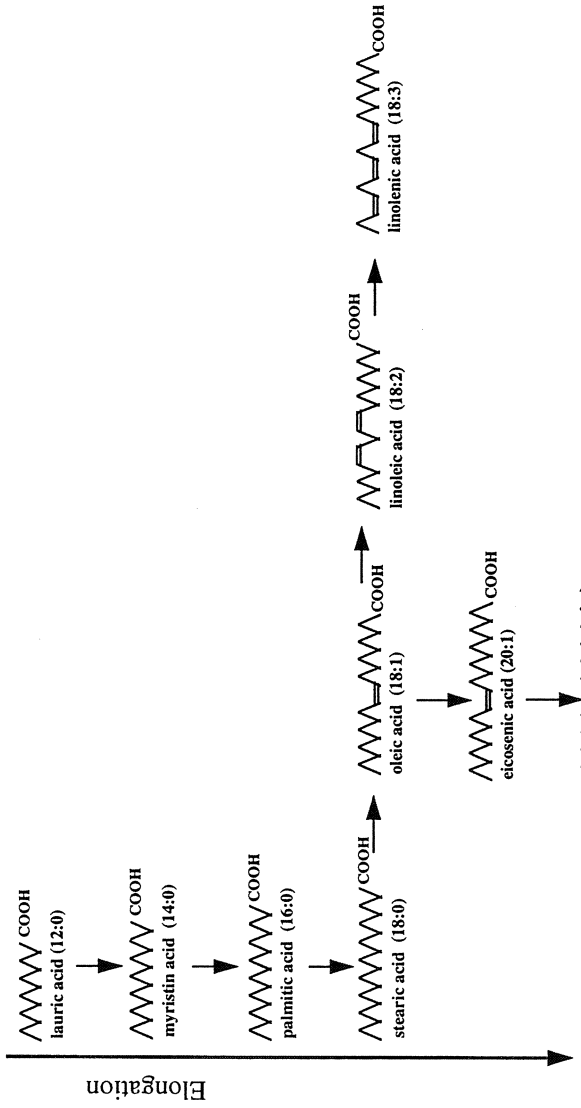

Figure 1. Biosynthesis of fatty acid in rapeseed

3. Directly selected, high oleic acid mutants. Recently, after mutagenic treatment of a large population of the variety Wotan a screening programme was carried out among more than 50.000 M_1 plant progenies within the SONCA research project [3], partly financed by the EEC. Chemical analyses were carried out in close cooperation in the research labs of the Institut für Pflanzenzüchtung in Göttingen, the INRA Plant Breeding Station in Rennes and of our company. Several mutants with a stable content of 70 % to 75 % oleic acid were finally identified after several cycles of selection [4].
4. Currently, transgenic materials are developed based on antisense constructs of desaturase enzymes and selection within these is directed to increased oleic acid contents.

The analytical screening and genetic selection has been rather labourious. The analyses of oleic acid content (table 2) was first carried out by gaschromatography (GC) only, but since 1992 „Near Infrared Reflection Spectroscopy (NIRS)" was introduced step by step [5]. The very high number of analyses in 1992 was due to the fact that in that year the analyses in the mutagen treated Wotan were carried out. Prerequisit for the reliable application of the NIRS-technique was an improvement of the calibration, which finally allowed the change over to this quicker, cheaper and non-destructive method. On the other hand, half-seed analyses, which in the breeding process is necessary for selecting single plants, can still be done by GC-analyses only.

Table 2. Number of analyses on oleic acid content

Year	GC-analysis	NIRS-analysis
1989	973	-
1990	1788	-
1991	1986	-
1992	11.314	3.432
1993	4.336	510
1994	1.338	230
1995	1.462	550
1996	2.905	3.415

Since 1996, new transgenic rapeseed lines with antisense desaturase constructs based on the constitutive promotor 35S have been analysed (table 3). As compared with the control variety Drakkar which already exhibits a slightly increased oleic acid content of 65.6 %, the oleic acid level of the transgenic material ranged between 70.0 % and 72.5 % only, which is a disappointing result! Nevertheless, selected single seeds (half-seeds) reached a maximum value of 76.7 %. New, improved transgenic materials contain the same antisense desaturase construct, but a seed specific promotor, and these have shown significantly higher values of about 85 % to 87 % oleic acid in first analyses. However, these data still lack confirmation in further generations.

Table 3. Percentage of fatty acids of transgenic rapeseed in the T3-generation after transformation with the E12 gene of desaturase in an antisense construct

Line	16:0	18:0	18:1	18:2	18:3	rest
Drakkar	4,2	1,2	65,6	17,8	9,0	2,2
HO 109/7*	4,3	1,7	70,7	12,6	6,8	3,9
HO 113/1	3,3	1,7	71,5	13,6	7,4	2,6
HO 113/2	4,5	1,9	70,2	13,8	7,0	3,3
HO 113/3	4,0	1,6	69,8	14,3	7,7	2,6
HO 115/2	3,4	1,9	70,0	14,7	7,3	2,6
HO 115/6	4,2	1,9	70,8	13,2	6,8	3,0
HO 115/14	3,5	1,8	70,0	14,5	7,6	2,5
HO 117/3	3,7	1,5	72,5	13,0	7,1	2,1

Progeny test by half seed selection:

Line	18:1	18:1 (half seed)		
	T3-plant	Ø	Min	Max
HO 109/7*	70,7	71,5	62,8	76,7

The presently given genetic variation for breeding HOAR will result in two different quality levels to become available in the near future (table 4). The „HOAR I"-type will exhibit a content of 75 % oleic acid and less than 3 % linolenic acid, whereas the „HOAR II"-type is designed to have above 90 % oleic acid and less than 2 % linolenic acid. It is obvious that the quality „HOAR I" can be bred by traditional techniques, whereas the quality „HOAR II" will require additional transgenic procedures.

Table 4. Fatty acid composition from different rapeseed genotypes

	Percent fatty acid of total lipids						
Type	16:0	18:0	18:1	18:2	18:3	20:1	22:1
High erucic	4,5	1,5	15,0	13,0	10,0	6,0	50,0
Low erucic	4,5	1,2	60,0	22,0	12,0	0,1	0,2
HOAR I	4,5	1,2	75,0	16,0	3,0	0,1	0,2
HOAR II	4,5	1,2	90,0	2,0	2,0	0,1	0,2
(transgenic, expected level)							

A key question for all applications of high oleic acid rapeseed varieties, in particular those for Biodiesel production, is the influence of this quality character on yield potential. Today no real figures on yield performance of these HOAR genotypes are at hand, but there are comparable results from the breeding materials selected for low linolenic acid [4]. Figure 2 presents the differences of relative seed yield of bulks selected for high and low linolenic acid content, respectively.

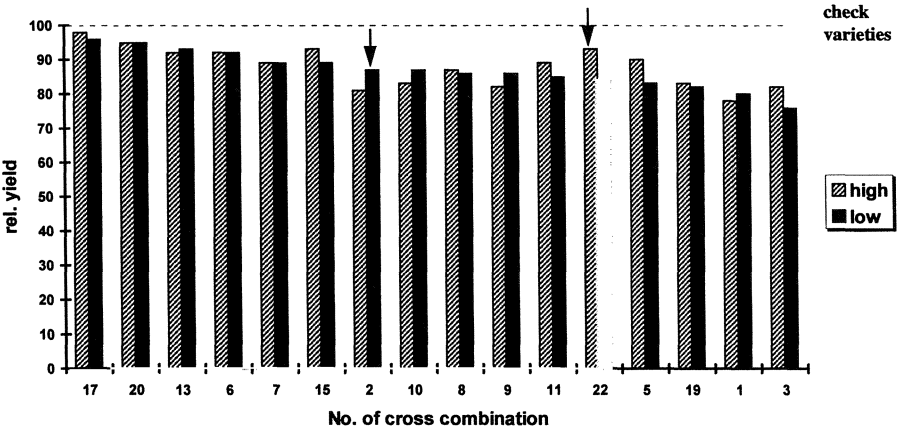

Figure 2. Relative seed yields of bulks selected for low vs. high linolenic acid content

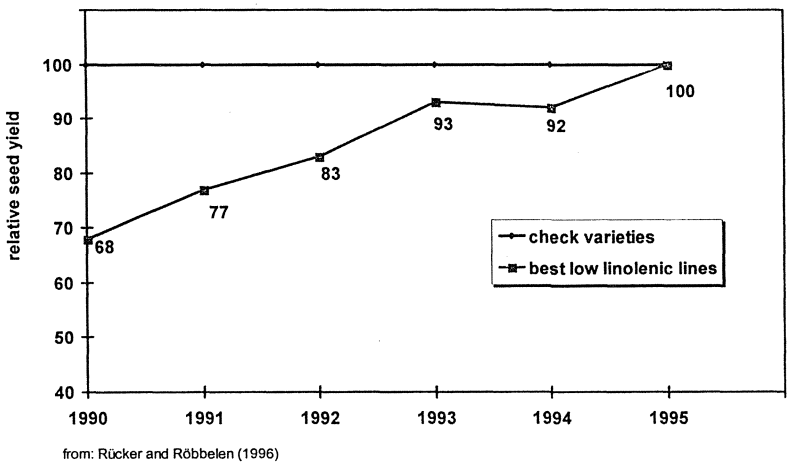

Figure 3. Comparison of best low linolenic lines (C18:3)

There are only small differences in the average between both bulk directions; in some cross combinations the low linolenic acid bulk (e.g. no.2) was higher in yield while vice versa in others the high linolenic acid bulk (e.g. no. 22) were superior in yield. Altogether the tested breeding bulks had less yield potential than the check varieties (with a relative yield of 100 %). This probably is due to the fact that all cross combinations contained the low linolenic acid parent with its original yield resulting in a segregation offspring with a lower yield average. However, the cross combination no. 17 almost reached the 100 % line of yield in both directions of selection.

A continuous yield improvement of low linolenic acid lines by recurrent backcrossing is shown during six years from 1990 to 1995 (figure 3). Finally the best lines reached the same seed yield level than the check varieties. Today hybrid varieties are being developed with 15 % to 20 % more yield than these check varieties. Consequently in future breeding cycles with the new low linolenic acid quality must be further backcrossed into different genepools and supplied with a sterility system in order to produce high yielding hybrid varieties. This will need another 5 to 6 years of breeding work.

Altogether, these data demonstrate, that the poorer yields of low linolenic lines and probably those of first HOAR lines, too, require a premium price for HOAR oil and HOAR varieties and, therefore, a higher price for the HOAR rapeseed harvested on the farmers fields, too. On the long run, however, yields similar to 00-varieties appear possible if this new quality is set to become a commodity product of rapeseed for different uses including Biodiesel.

References

1. G. Rakow, *Z. Pflanzenzüchtung* **69**, 62-82 (1973).
2. G. Röbbelen and A. Nitsch, *Z. Pflanzenzüchtg.* **75**, 93-105 (1975).
3. B. Rücker and G. Röbbelen, Mutants of brassica napus with altered seed lipid fatty acid compostion. *Proc. 12th Int. Symp.Plant Lipids.* July 8-12. 1996, Toronto, Canada. Kluwer Acad. Publ., Dordrecht, The Netherlands, in press (1996a).
4. B. Rücker and G. Röbbelen, *Plant breeding* **115**, 226-230 (1996b).
5. T.-C. Reinhardt, thesis, FB Agrarwiss., Univ. Göttingen (1992).

Genetic Engineering of *Brassica napus* Seeds for Oil Quality and Yield with Toxicological and Motor-Technical Aspects of Biodiesel in Mind

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In the temperate climate of Europe rapeseed (*Brassica napus*, figure 1A) is the most important oil crop followed by sunflower and linseed. Its storage oil is made up of triacylglycerols, consisting of a glycerol moiety esterified to three fatty acyl residues with a chain length of predominantly 18 to 22 carbon atoms.

Figure 1. **A:** *Brassica napus*, **B:** *Cuphea lanceolata* (photographs by M. Kalda)

In the past, conventional breeding of rapeseed resulted in two main categories of high-yielding varieties: high erucic acid cultivars of rapeseed accumulating predominantly erucic acid, a very long chain fatty acid (C22:1), and double-low

varieties with over 95 % of the seed oil composed of only five C16 and C18 fatty acids (i.e palmitic, stearic, oleic, linoleic and alpha linolenic acids) and almost no erucic acid besides a low glucosinolate content. Typical fatty acid profiles of rapeseed are depicted in table 1. Concommitant scientific observations led to the conclusion that the amount of erucic acid present in *Brassica* seed oils is controlled by the genetic design of the developing embryo, rather than the maternal parent [1]. These findings therefore revealed the potential of this crop for significant inheritable changes in oil quality.

Table 1. Two examples of fatty acid composition of rapeseed oil (wt %)

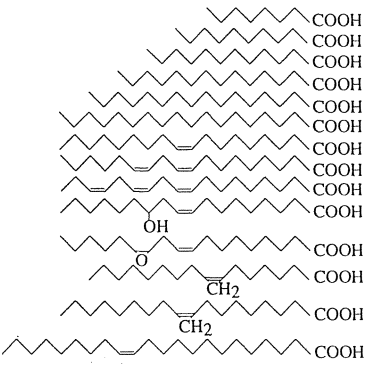
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
++	3	1	16	14	10	1	6	tr	49
00	4	2	56	26	10	tr	2	tr	tr

++ High-erucic-acid rapeseed
00 Double-low rapeseed (low in erucic acid and low in glucosinolate)
tr = traces [2]

Fatty acids from plants vary in their chain length and in the number and position of double-bonds, hydroxy and epoxy groups and carbon branches. There are plant species in whose seed oil a single fatty acid makes up to 90 % of all fatty acids. For instance, castorbean has 90 % ricinoleic acid (C18:1-OH), *Cuphea pulcherrima* has 94 % caprylic acid (C8:0), and *C. lanceolata* has 83 % capric acid (C10:0). Typically, different *Cuphea* species contain an unusual high amount of a particular medium-chain fatty acid in their seed storage lipids [3]. Table 2 provides an overview of special quality oils found in wild species or in oil crops.

Table 2. Naturally occurring diversity of oil composition in plant seeds (2)

Plant Species	Amount of Total (%)	Predominant Fatty Acid	Fatty Acid Structure and Chain Length
Cuphea pulcherrima	94	Caprylic acid	
Cuphea lanceolata	83	Capric acid	
Elaeis guineensis (kernel)	49	Lauric acid	
Cuphea palustris	64	Myristic acid	
Elaeis guineensis (mesocarp)	47	Palmitic acid	
Theobroma cacao	34	Stearic acid	
Brassica napus (00)	62	Oleic acid	
Gossypium hirsutum	53	Linoleic acid	
Linum usitatissimum	60	Linolenic acid	
Ricinus communis	90	Ricinoleic acid	
Vernonia roxburghii	75	Vernolic acid	
Gnetum gnomen	39	Malvalic acid	
Sterculia foetida	65	Sterculic acid	
Brassica napus (++)	49	Erucic acid	



In principle, each genetically controlled trait of a given plant whether in wild species or in a crop can be transferred with the ultimate goal of designing

transgenic oil crops with tailor-made fatty acid compositions. A major contribution of modern biotechnology to rapeseed breeding will be the transfer of genes responsible for the formation of short or medium-chain fatty acids (C8:0 to C14:0) or fatty acids with defined functional groups especially as homogeneous compounds in oils from transgenic rapeseed varieties. Thus, it is essential to uncover the underlying molecular mechanisms and their controlling genes in order to efficiently transfer these traits to transgenic oil crops. As prerequisites to these genetic approaches the isolation of relevant genes and the availability of appropriate regulatory sequences directing transgene expression to the oil storage tissues are therefore indispensible.

As an example, a project at the Max-Planck-Institute is briefly described aiming at the production of capric acid in rapeseed oil, a goal which is unattainable by conventional breeding methods. The gene source is *C. lanceolata*, a wild species from Central America (figure 1B). It has several features which render the plant itself unsuitable for cultivation and ultimate exploitation of its oil. The plant's indeterminate growth pattern results in uneven seed ripening. Moreover, it exhibits an effective seed dispersal mechanism, and the plant is covered by a gluey exudate. Nevertheless, attempts were made to domesticate *Cuphea* species as a crop with the aim of providing a new source for medium-chain fatty acids [4].

Advances in our knowledge about the biosynthesis of plant fatty acids have been recently reviewed [5]. The basic mechanism for the accumulation of medium-chain fatty acids in plants is schematically depicted in figure 2.

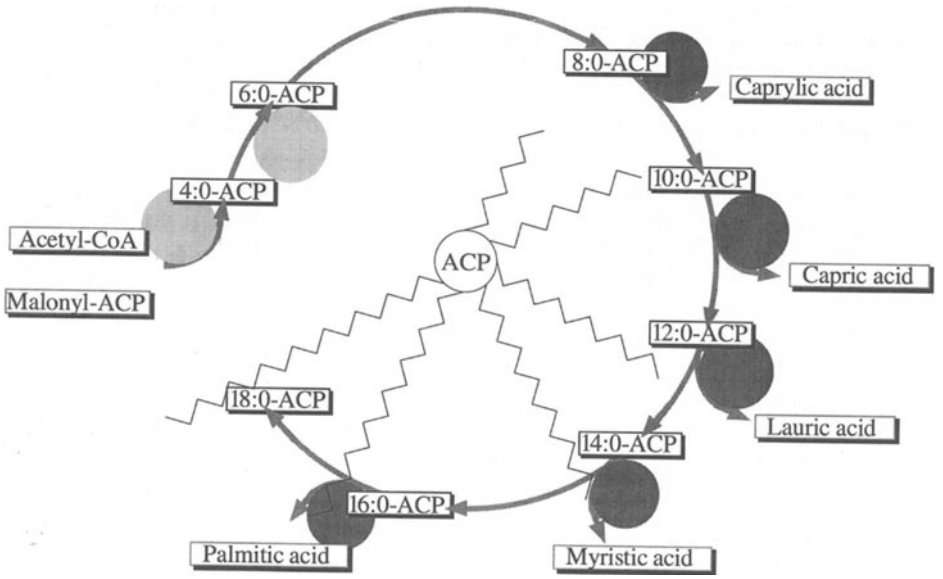


Figure 2. Basic mechanism for the production of medium-chain fatty acids in plants (schematic drawing explained in the text)

The initial reaction in the formation of fatty acids is the condensation of the starter molecule acetyl-coenzyme A (acetyl-CoA) with malonyl-acyl carrier protein (malonyl-ACP) to yield C4:0-ACP. A complex of different enzymes (light dots) is involved in preparing the ACP-bound substrate for a subsequent condensation with malonyl-ACP. Each condensation step elongates the acyl chain by two carbons as long as it is bound to its carrier ACP. Specific fatty acyl-ACP thioesterases (Fat) catalyze either the hydrolysis of the final condensation product, oleoyl-ACP (FatA, not shown) or the premature hydrolysis of the growing acyl chain (FatB, dark dots), thus releasing the free fatty acid with a specific chain length from its carrier. FatA is specifically active in rapeseed, for instance, whereas FatB activity is predominantly found in seeds of plants accumulating medium-chain fatty acids (see M. Sovero's chapter).

We isolated four members of the FatB gene family from *C. lanceolata*. When two of these genes responsible for embryo-specific expression in *C. lanceolata* [6] were transferred and expressed in transgenic rapeseed lines, distinct altered fatty acid profiles were observed in mature T2-seeds. One FatB gene conferred the production of 6 mol % capric acid, while the transfer of the other yielded 23 mol % myristic and 29 mol % palmitic acid [7]. Together with the results obtained elsewhere [8] there is now a portefolio of transgenic rapeseed lines/varieties which accumulate a whole spectrum of saturated fatty acids of various chain lengths. A summary of recent achievements is depicted in figure 3. We conclude that the addition of specific FatB genes allows for the transfer of hereditary metabolic properties from wild organisms (e.g. *Cuphea*) to an oil crop with rapeseed being a model crop, and with the prospect that other oil crops will follow.

Reduction of an enzyme activity was achieved by antisense-repression of the delta 9 desaturase gene in transgenic varieties. Up to 50 % stearic acid accumulated in their seed oil while its desaturation to oleic acid was blocked [8]. Similarly, addition of specific genes e.g. the oleate hydroxylase gene from castorbean, or the acyl-CoA reductase gene from jojoba as well as suppression of an endogenous delta 12 desaturase gene led to the accumulation of yet different lipids in plants: 17 % ricinoleic acid in *Arabidopsis thaliana* [9], 15 % liquid wax [8] and up to 88 % oleic acid [6], respectively (figure 3). In summary, these more or less preliminary results demonstrate pretty well that it is now possible to transfer hereditary metabolic properties from one organism to another, and by doing so to modify the fatty acid composition of rapeseed oil in a predictable manner. Future research and development in oilseed biotechnology can be counted on to yield even more desirable fatty acid compositions.

In order to approach the goal of producing more than 90 % of a particular fatty acid in an oil crop, the study of respective plant species whose seed oil comprises up to 90 % of a single fatty acid, like castorbean or *Cuphea pulcherrima*, should help paving the way. The occurrence of these plants and the different molecular species of fatty acids which are stored in their seeds in high percentages mean that there is no basic physiological reason which would prevent seeds from accumulating 80-90 % of a particular fatty acid in their oil. It remains for biochemists and molecular geneticists to uncover the underlying mechanisms and their controlling genes so that these traits can be efficiently transferred to transgenic oil crops.

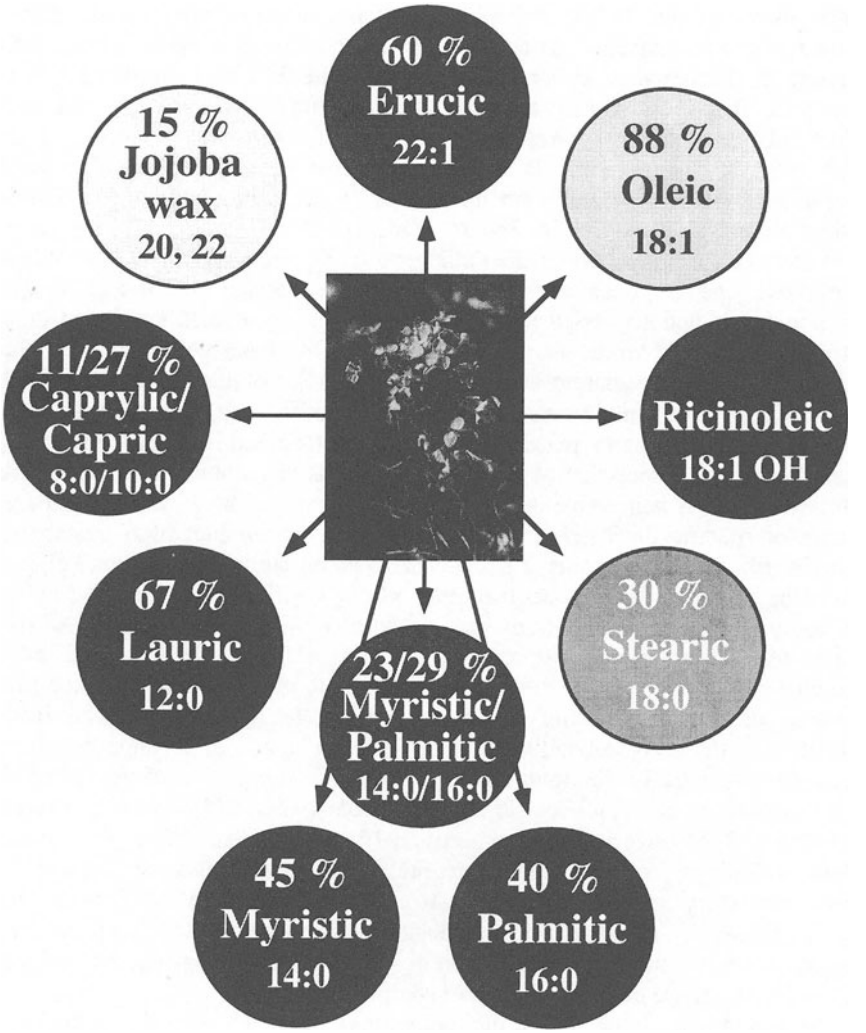


Figure 3. Modification of lipid synthesis in rapeseed

Two targets with respect to medium-chain-length specificity have already been identified and adressed in a joint project funded by the German Federal Ministry of Education and Research (BMBF) and German plant breeders: Firstly, *in vitro* biochemical investigations with *C. lanceolata* seed extracts revealed a specific condensing enzyme which barely elongates C10:0-ACP, leading to an increase in

this metabolite pool which is ultimately processed by the specific FatB gene product to release free C10:0 fatty acids [10]. A second target is a membrane-bound acyltransferase in the endoplasmic reticulum, which mediates the transfer of activated fatty acyl residues to the middle position of the glycerol molecule during the assembly of triacylglycerols. Since in rapeseed this enzyme is highly specific for C18 unsaturated fatty acids a medium-chain specific acyltransferase from *C. lanceolata* needs to be transferred to rapeseed.

In order to proceed towards making rapeseed oil methyl ester (Biodiesel) a realistic proposition toxicological, motor-technical and economical aspects must be taken into account. Here is what is requested and how we propose one could contribute:

– Further reduction of soot and particulate matter:

The relative proportion of oxygen to carbon in fuel compounds influences the emission of soot and particulate matter as a consequence of a more or less complete combustion process. For instance, dimethyl ether with an intrinsic oxygen content of about 40 % was shown to drastically reduce emission of soot and particles in the exaust of diesel engines [11]. Biodiesel contains 10 % oxygen leading to a reduced emission of soot and particulate matter, whereas petroleum-based diesel (petrodiesel) has 0 % oxygen. Rapeseed oil developed for the production of short-chain fatty acids like capric acid (C10:0) and/or caprylic acid (C8:0) will provide a higher proportion of oxygen to carbon than C18 unsaturated fatty acids simply due to a reduced carbon number. Thus, a further reduction of soot and particles in the exhaust of this liquid fuel might be expected. Methyl esters of capric acid or caprylic acid have melting points comparable with or lower than methyl oleate (C18:1-CH3, figure 4), a major compound in conventional Biodiesel. Whether an oil with a high C8:0 or C10:0 content will meet toxicological and/or motor-technical requirements when used as Biodiesel fuel needs further investigation. Yet, one should bear in mind that this type of oil has a lower caloric value. It should here also be notified that a particular fatty acid melting point is not correlated with the cold filter plugging point (see W. Körbitz's chapter) of a liquid fuel.

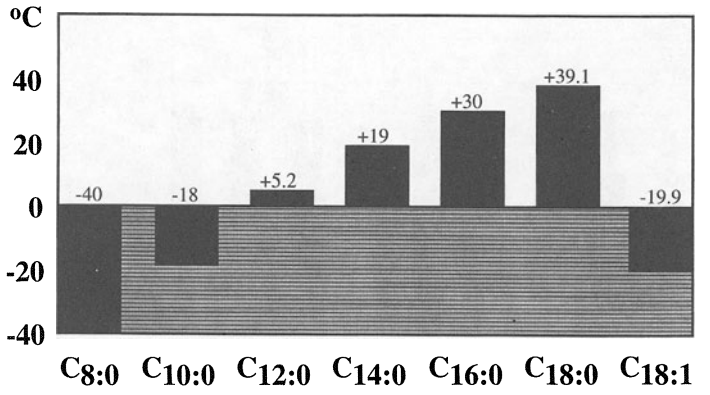


Figure 4. Melting points of the methyl ester of fatty acids (12)

– Sulfur content:

The sulfur content is already drastically reduced in Biodiesel as compared with petrodiesel. Most likely the remaining sulfur in rapeseed oil results from sulfolipids which are extracted together with the oil. Sulfolipids are integral compounds of thylakoid membranes in plastids. 5 % of thylakoid lipids in chloroplasts consist of sulfoquinovosyl diacylglycerol. Thus, this lipid class is probably indispensible to living organisms. The nature of the headgroup donor and the means of its synthesis are still unknown. Further investigations are required.

– Reduction of phosphorus content:

In Biodiesel phosphorus most likely stems from membrane phospholipids, which are indispensible to living organisms. As yet, there is no obvious clue as to the way to reduce the phosphorus content in rapeseed oil other than by chemical separation. One could however attempt to increase the ratio between storage oil and phospohorus content derived from membranes.

– Reduction in production costs:

80 % of the monetary value of the seed is derived from the extracted oil. Therefore an important goal in plant breeding is increase in oil yield. One factor influencing oil yield is the oil content per seed. Although correlations between seed yield and oil content have not been observed, the heritability of oil content is higher than for seed yield [1]. Since traditional breeding has already shown, that it is possible to increase the oil yield in high-yielding oil crops, it must be possible to further increase the oil content in these crops by means of genetic engineering.

Most of the major oilseed crops like soybean, rapeseed, or sunflower contain about 20-45 % (w/w) oil on a dry-weight basis in their seeds [13]. However, there are numerous species that contain 60-70 % (w/w) oil in their seeds (figure 5). The South-American Ucuhuba plant, for instance, contains 65-76 % oil in hazelnut-size seeds [2]. The observed variation in seed oil content of different species leads us to conclude that there is no fundamental biological reason why the content of certain seed oils in oil crops cannot be increased to almost double the present level. For instance, rapeseed contains twice as much oil as soybean (figure 5) simply due to the fact that it can synthesize oil twice as quickly during seed development [14].

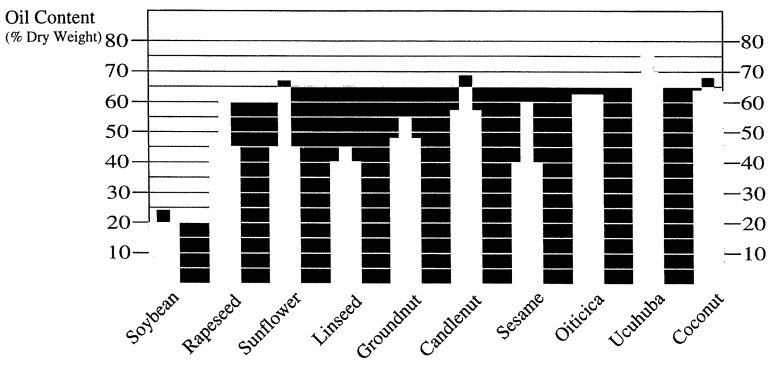


Figure 5. Oil content in seeds of various plants (2, 13). Grey bars represent mean values, and black bars indicate maximal values which have been observed so far.

Since all seeds can make oil, protein, carbohydrate, and fibre, the higher content of one component relative to the others is a matter of the proportion of carbon that is partitioned to any particular end-product. Acetyl-coenzyme A (acetyl-CoA), the key metabolite at the interface between catabolism and anabolism in plants, serves as immediate substrate in the enzyme reaction committed to *de novo* fatty acid biosynthesis in plastids. One could therefore attempt to enhance the carbon flux towards oil by modifying rate-limiting steps involved in oil production. Two enzymes previously suggested as possible bottlenecks for *de novo* fatty acid biosynthesis and oil accumulation are acetyl-CoA carboxylase (ACCase) and ß-ketoacyl-ACP synthase III (KAS III), the latter one being a key player in the initial condensation step leading to C4:0-ACP [15]. The extent of metabolite flux through a given metabolic pathway is dependent on the activities of the individual enzymes involved. Therefore, modification of the genetic control over a rate-limiting step can be achieved by altering the amount of enzyme present in defined cells (spatial control) and at a given time during tissue differentiation (developmental control).

A project at the Max-Planck-Institute deals with the spatially and developmentally controlled overexpression of the ACCase gene in seeds of rapeseed with the aim to feed the fatty acid biosynthetic machinery with more substrate hopefully leading to more deposited oil. ACCase is a key enzyme in *de novo* fatty acid biosynthesis which is localized in plastids. The ATP-dependent conversion of acetyl-CoA to malonyl-CoA proceeds in two steps:

(1) $ATP + HCO_3^- + BCCP \xrightarrow{\text{Biotin carboxylase}} ADP + P_i + COO^-\text{-}BCCP$

(2) $COO^-\text{-}BCCP + \text{Acetyl-CoA} \xrightarrow{\text{Carboxyl transferase}} BCCP + \text{Malonyl-CoA}$

At the expense of adenosine triphosphate (ATP) bicarbonate (HCO_3^-) is transferred to a biotin prosthetic group which is covalently attached to its carrier protein (BCCP) resulting in carboxybiotin. From this intermediate the activated carboxyl group (COO^-) is ultimately transferred to acetyl-CoA resulting in malonyl-CoA. The proposed approach towards an increase in carbon flux in order to provide more substrate for fatty acid biosynthesis should affect the amount of all subsequent products whether short-, medium- or long-chain fatty acids which leave the plastid for incoporation into triacylglycerols.

Besides fatty acid synthesis malonyl-CoA is also required in fatty acid elongation, in the synthesis of flavonoids and stilbenoids and in the malonation of various compounds. In plant cells there is a need for malonyl-CoA in plastids as well as in the cytosol. Thus, at least two isoformes of ACCase are required to meet the various needs for malonyl-CoA.

We isolated several genomic ACCase clones from rapeseed which were divided into two major distinct classes [16]. Comparison of ACCase sequences from *B.napus*, *Arabidopsis thaliana*, alfalfa and wheat with each other, revealed a novel feature in the region around the translation start site of class 1 ACCases from the

dicotyledonous *B. napus* (figure 6). Class 1 ACCase possesses an additional coding exon, which is absent in class 2 ACCase of rapeseed, as well as in the other thus far published plant ACCase sequences, which are represented in figure 6 by the genomic equivalent of an Arabidopsis ACCase. Since the latter ones all lack characteristic features of transit peptides, they have been proposed to encode enzymes localized in the cytosol, which is in agreement with the afore-mentioned requirements for malonyl-CoA in cytosolic pathways. However, the first exon of class 1 ACCase of rapeseed has indeed features of transit peptides. Thus, ACCase appears to be the largest polypeptide (approximately 220 kDa) which can be transported into plastids. Moreover, class 2 ACCase from rapeseed contains an additional intron of 560 bp in the 5'untranslated region (figure 6).

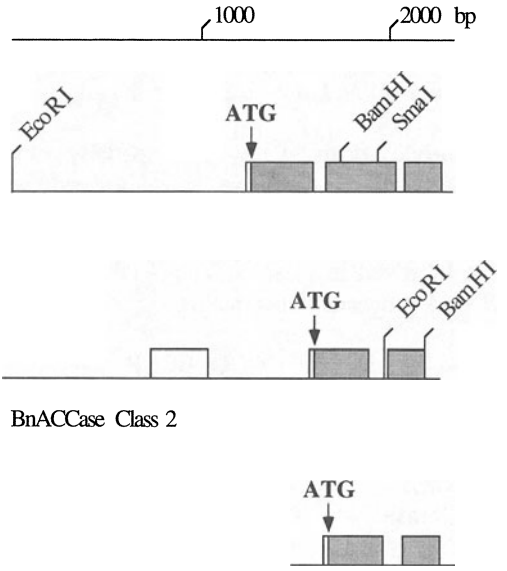


Figure 6. Two distinct classes of the multi-functional ACCase in *B. napus* (*Bn*). Schematic representation of the characteristic regions around the translation start site (ATG) of the genes is depicted. EcoRI, BamHI, and SmaI indicate restriction sites. Grey and white boxes represent coding and non-coding exons, respectively. *AtACCase* = multi-functional acetyl-CoA carboxylase from *Arabidopsis thaliana* (17).

The function of the putative transit peptide sequence in the first exon of rapeseed ACCase (class 1) was confirmed *in vivo* via transient expression in tobacco protoplasts. A fusion between the corresponding piece of DNA and a reporter gene coding for the green fluorescent protein (GFP) indeed targeted GFP to the chloroplasts. figure 7 shows the implications of this finding in rapeseed. The GFP experiment indicates that a multi-functional ACCase is localized in plastids of rapeseed in addition to multi-subunit components [16]. Until recently it was believed that in dicotyledonous plants such as rapeseed the multi-functional

ACCase resides in the cytosol and the multi-subunit type is located in the plastids. In Gramineae, however, the multi-subunit form seems to be absent and isozymes of the multi-functional type are found in both cellular compartments [18].

A short summary of biochemical studies explains the difference between multi-subunit and multi-functional isoformes as outlined in figure 7. The multi-subunit form of ACCase was found in procaryotes first and consists of three dissociable components comprising a dimer of two 17 kDa biotin carboxyl carrier protein subunits, a dimer of two 49 kDa biotin carboxylase subunits and a 130 kDa carboxyl transferase complex made up of two pairs of non-identical 35 kDa and 33 kDA subunits. The organisation of the multi-functional form of ACCase is different. ACCases of all plants and animals examined to date, were shown to consist of one or more multi-functional subunits, each of approximately 220-280 kDa. There are 4 multi-functional subunits in yeast, multiple subunits in animals and 2 subunits in plants. Each subunit contains a biotinylated domain, a biotin carboxylase and a carboxyl transferase domain. In 1995 reports were published providing evidence for the existence of a multi-subunit form of ACCase in some plants (so far only in dicots) [18].

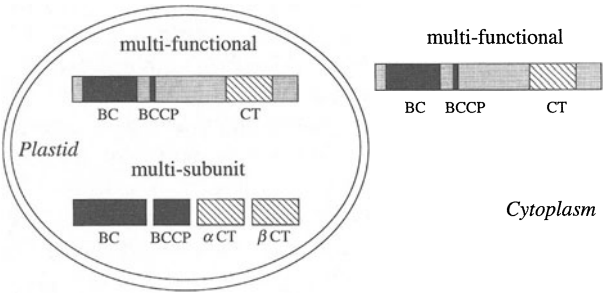


Figure 7. Occurrence of multi-functional and multi-subunit ACCase in rapeseed (schematic). BC = Biotin Carboxylase, BCCP = Biotin Carboxyl Carrier Protein, CT = Carboxyl Transferase

In animal cells ACCase is a major rate-limiting enzyme of fatty acid biosynthesis. The enzyme is subjected to strong metabolic control by phosphorylation and dephosphorylation, which is affected by the nutritional status of the animal. Citrate and long chain acyl-CoA were demonstrated to act as metabolic effectors of the polymerisation/depolymerisation status of ACCase thus affecting its activity.

In plants little is known about the metabolic control of ACCase activity. In tobacco suspension culture cells, plastidic ACCase was found to be tightly regulated by mechanisms including feedback inhibition [19]. If feedback control

of ACCase in rapeseed embryos regulated the velocity of its catalytic activity, the effectors would most likely be of different nature in the two cellular compartments, plastid and cytosol. Should this be the case, the most likely regulators in plastids would be long-chain fatty acyl residues, whereas in the cytosol of rapeseed embryos one would expect very-long-chain fatty acyl residues to play this role. Targeting of cytosolic ACCase to plastids and/or of plastidic ACCase to the cytosol would be a realistic approach to circumvent fine metabolic control. For instance, an increase in starch accumulation was observed in potato with a bacterial mutant gene, defective in feedback regulation and coding for the rate-limiting enzyme in starch synthesis, but not with the overexpression of the endogenous plant gene [20].

In order to re-introduce the ACCase gene under alternative genetic control, various promoters known to work specifically in rapeseed embryos have been selected. A fatty acyl-ACP thioesterase promoter which is derived from a gene involved in fatty acid biosynthesis in *C. lanceolata* might be a good candidate to achieve maximal ACCase expression. Other regulatory sequences are promoters of seed-specific napin [21] and oleosin genes [22] and the 35S-rna promoter, which drives gene expression throughout the plant [23]. ACCase gene fusions to these promoters were constructed and are currently being transferred into rapeseed [24].

The feasibility of this approach was recently supported by an approximately 5 % increase of the total oil content of seeds from transgenic rapeseed upon expression of a multi-functional ACCase from *Arabidopsis thaliana* targeted to the plastids. In addition, the plastid localization of the heterologous ACCase appears to prevent its turnover and to escape a presumed feedback inhibition [25].

Recently, Canadian scientists reported that a yeast acyltransferase gene was expressed in rapeseed with the initial intention to increase the erucic acid content by specifically mediating the transfer of erucoyl-CoA to the normally scarcely occupied middle-position of triacylglycerols by this fatty acid species. Instead of increasing the overall erucic acid content, the expressed yeast enzyme resulted unexpectedly in a significant increase in oil content. On a dry weight basis, the transgenic seeds contained up to 22 % more oil as compared with the control [26]. Overexpression of the heterologous enzyme localized way down-stream in the biosynthetic pathway leading to oil formation thus appeared to overcome biochemical control mechanisms including feedback control, which are presumably shared among several enzymes in the pathway. The relatively small increase in seed oil due to enhanced ACCase activity in seed plastids of transgenic rapeseed may have been limited by other enzymatic steps in the pathway. Thus, crossing the two engineered lines with increased seed oil content might be a promising approach for further increases.

If scientists understand the genetic and biochemical control mechanisms regulating an increased oil content over the other storage compounds, starch and protein, and have the responsible genes at hand, it should be possible to increase the oil content of rapeseed by 100 %.

Conclusion

The applications of gene transfer technology to agriculture is limited only by our understanding of the biochemical mechanisms and the underlying genetic principles responsible for crop quality and production. Only once these fundamental mechanisms are understood, can geneticists, molecular biologists, and breeders fully optimize conditions which will lead to increases in crop yields. The examples offered in this chapter serve to illustrate the potential of molecular breeding. In principal, each genetically determined trait can be transferred to rapeseed. Once we will have learnt to transfer more effectively and more extensively particular oil-related traits of interest to rapeseed including varieties for the production of Biodiesel, one can later target other oil crop plants, especially those relevant to the developing countries. It is tempting to envision plants which serve as bioreactors for the supply of various industrial needs, perhaps some time including liquid esters as one form of energy amongst others.

Acknowledgement

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References

1. R. K. Downey and G. Röbbelen, in *Oil Crops of the World*, G. Röbbelen, R. K. Downey, and A. Ashri, Eds. (McGraw-Hill, New York, 1989), p. 339-362.
2. F. D. Gunstone, J. L. Harwood, F. B. Padley, *The Lipid Handbook* (Chapman and Hall, London, ed. 2, 1994).
3. S. A. Graham, *Crit. Rev. Food Sci. Nutr.* **28**, 139 (1989).
4. S. J. Knapp, *Plant Breed.* **104**, 46 (1990).
5. J. L. Harwood, *Biochim. Biophys. Acta* **1301**, 7 (1996).
6. R. Töpfer, N. Martini, J. Schell, *Science* **268**, 609 (1995).
7. N. Martini, R. Töpfer, J. Schell, unpublished.
8. An update of engineered oil qualities in rapeseed by Calgene Inc. Davis, Ca. was kindly provided by M. Sovero.
9. P. Broun and C. Somerville, *Plant Physiol.* **113**, 933 (1997).

10. R. Schuch, M. Brummel, F. Spener, *Grassas y Aceites* **44**, 126 (1993).
11. P. Kapus and H. Ofner, *Society of Automotive Engineers Technical Paper* 950062 (1995).
12. D. R. Lide, *CRC Handbook of Chemistry and Physics* (CRC Press Inc., Boca Raton, ed. 74, 1993).
13. W. Lühs and W. Friedt, in *Designer Oil Crops*, D. J. Murphy, Ed. (VCH, Weinheim, Germany, 1993), p.5.
14. D. J. Murphy, *Trends in Biotechnology* **14**, 206 (1996).
15. J. B. Ohlrogge, J. G. Jawaorski, D. Post-Beittenmiller, in *Lipid Metabolism in Plants*, T. S. Moore, Ed. (CRC Boca-Raton, 1993), p. 3-32.
16. W. Schulte, R. Töpfer, R. Stracke, J. Schell, N. Martini, *Proc. Natl. Acad. Sci. USA* **94**, 3465 (1997).
17. K. R. Roesler, B. S. Shorrosh, J. B. Ohlrogge, *Plant Physiol.* **105**, 611 (1994).
18. Y. Sasaki, T. Konishi, Y. Nagano, *Plant Physiol.* **108**, 445 (1995).
19. D. K. Shintani and J. B. Ohlrogge, *Plant J.* **7**, 577 (1995).
20. D. M. Stark, K. P. Timmerman, G. F. Barry, J. Preiss, G. M. Kishore, *Science* **258**, 287 (1992).
21. J. C. Kridl *et al.*, *Seed Sci. Res.* **1**, 209 (1991).
22. J. S. Keddie *et al.*, *Plant Mol. Biol.* **24**, 327 (1994).
23. J. T. Odell, S. Knowlton, W. Lin, C. J. Mauvais, *Plant Mol. Biol.* **10**, 263 (1987).
24. W. Schulte, J. Schell, N. Martini, unpublished.
25. K. Roesler, D. Shintani, L. Savage, S. Boddupalli, J. Ohlrogge, *Plant Physiol.* **113**, 75 (1997).
26. J. Zou *et al.*, *The Plant Cell* **9**, 909 (1997).

Discussion of Session 3: Contribution of Plant Breeding for Making Biodiesel Rapeseed a Realistic Proposition

Presentation by **G Kley**: *Breeding of Hybrid Rapeseed.*

Presentation by **J Botterman**: *Pollination Control in Transgenic Rapeseed: from Concept to Market Entry of F1 Hybrids.*

J Schell: Dr Botterman, what is the basis of your optimism that higher yields can be obtained from hybrid crops?

J Botterman: The basis of this optimism is oil seed rebreeding. We have been quite active in assembling germplasm from private industry and from public institutes. Since a start has to be made somewhere, we have been making random combinations and a hybrid potential is already apparent. With a more rational approach using molecular and molecular-assisted selection and further development to improve female and male blocks considerable progress will be made. Whilst the real potential can't be quantified, more than 10–15 % is certainly possible. At the same time there are already effects in terms of heterotic potential.

J Schell: The breeders might comment on this. Dr Kley said 10–15 %, Dr. Botterman said more than 10–15 %.

G Kley: For the time being 15–20 %, without having used the specific combining ability. This is based on general combining ability. We need to produce more experimental lines to achieve more specific combining ability, then it will certainly exceed 20 %.

G Röbbelen: There is no reason not to accept this optimism. The percentages shouldn't be overemphasised. But the literature on corn shows much higher heterosis percentages, 50 % or so. This is, however, not relevant because heterosis is the surplus of the hybrid over the parental performance and in corn breeding the parental performance is that of inbred lines with all inbreeding deficiency included. For rapeseed, the parental performance is the performance of our normal conventional varieties and over these varieties, 10–15 % may represent more than a hybrid heterotic increase of 30 % in corn. What one really needs to know is the hybrid performance, and not the heterotic percentage. Certainly, random hybrids have been made that are available and that could be selected from the given

material, but the directed improvement of the lines and gene pools for hybrid combining ability is still much in advent.

M Frauen: The bottom-line 100 % will increase. All open pollinating (OP) varieties and the best hybrids should be set at 100 % and , this will increase in the next years, because breeders continue to breeding conventional varieties. The gap between the best OP varieties and hybrids may be 20–25 %. So whilst there will be a 40 % yield increase above today's total level, the bottom-line will increase as well. There is a type of battle between the conventional OP variety breeding and the hybrid breeding. Genetic distance is one factor for achieving a high hybrid yield; the other is the yield per se of the inbred lines. Thus corn breeding also shows that the yield per se has been increased on the inbred lines much more efficiently than by heterotic effects.

F Salamini: My question is to Dr Kley: It is clear that as soon as a hybrid project is started, 10–20 % increases are obtained. This increase must then be maintained during the conventional breeding period and you cited progress of 1–3 %. For maize, the most intensively studied plant, usually 1 % per year is reckoned for a 50-year period. The effort for maize is really intense; one company alone in the USA cover more than 2 Mio. plots per year in temperate areas of the world. Are you really sure about the 1–3 %? Is the breeding effort so consistent that a 1–3 % yield increase will double the yield per hectare by the year 2010?

G Kley: Yes, because this has been already achieved. We have obtained a 1–5 % increase in producing new conventional OP varieties. If we focus on creating new lines as a basis for future hybrid breeding, why should this not increase to the same extent? In addition, the enhancement of disease resistance can be expected to be supported by biotechnical means, which is a completely new development. For the time being, only those genetic resources of disease resistance that are linked to internationally available normal material can be used, but in the future traits originating from other crops or other species and which enhance disease resistance can be used. Thus a 1–5 % annual increase in yield, as obtained in the past, is a realistic perspective for the future.

J Schell: To conclude, a 100 % increase in yield is a goal that both traditional breeders and molecular biologists are working on and consider to be achievable.

Presentation by **E Rasche**: *Rapeseed with Tolerance to the Non Selective Herbicide Glufosinate Ammonium.*

Presentation by **C Maas**: *Plant Biotechnology and Implications for Rapeseed Agronomy: Development of New Methods of Pest and Disease Control.*

K Thurau: Making plants disease resistant is certainly a very important development. My question is: might the development of this resistance favour the modification of bacteria and viruses so as to render the defence mechanism ineffective again?

C Maas: This would be a situation very similar to that with which breeders are already familiar. The combination of different resistance strategies is a means to reduce the probability of emergence of resistance-breaking strains.

E Rasche: Important in this regard is the need to combine different means to maintain good control. "Different means" means the use of chemical control in addition to resistant crops. Take, for example, *B.t.* cotton (resistant against cotton bollworm). The possibility has to be faced that if just the one principle is employed extensively, resistance will develop against it and will break it down. With the chemical control systems, like insecticides or fungicides which have long been available, this has happened frequently, especially when modern molecules are used. Their particular mode of action can be more easily overcome by a single developmental step of insects or diseases. There is much experience available to industry with regard to resistance management. We therefore talk about integrated pest management programmes, where integrated means the use of all possible means to overcome the problems. This will be true also for transgenic plants that resist diseases or insects.

Presentation by **M Sovero**: *Modification of Fatty Acids by Genetic Engineering.*

Presentation by **M Frauen**: *Breeding of High-Oleic-Acid Rapeseed (HOAR).*

J Schell: Professor Röbbelen, since we have seen data from your laboratory, would you like to comment?

G Röbbelen: Dr Frauen said that there is no yield penalty at the end—and this is my comment—many mutants showed a variation in fatty acid composition in the desired direction in their seed oil but also, for example, in the membranes of their roots; these, of course, have to be discarded; the individual mutant usually is far more complex than could be explained in my short review.

J Schell: Would this possibly be an argument for using genetic engineering in addition to traditional breeding?

M Frauen: Yes. The desaturase antisense constructs could be more efficient in that they don't have the draw back in the yield potential. And since the antisense desaturase constructs are controlled by seed-specific promoters, this will be a better solution for this breeding approach. However, both approaches, genetic engineering and traditional breeding need to be combined. Transgenic variation alone may not generate the desired extremely high oleic acid rapeseed lines.

W Körbitz: What is the level of saturated fatty acids?

M Frauen: The level of saturates has not changed; we observe 6 % even in this new material.

W Körbitz: The high oleic-type oil is a better raw material, not just for Biodiesel but for other technical purposes, too. I hope the price will not be too high.

G Kley: May I draw your attention to the cost efficiency of breeding progress. The variable costs per ha are DM 1,000–1,200 and are roughly stable regardless of yield. For an actual average oil yield of 1.4 t/ha, this amounts to DM 857/t. For a future yield of about 2.9 t/ha, the production cost will be 52 % lower (DM 413). This is a price which is currently traded internationally. This shows that it is very important to improve competitiveness for oil when we reach an oil yield double that of today. This is an example of how breeding progress really affects positively the cost effectiveness of producing vegetable oil for industrial use.

Presentation by **N Martini**: *Genetic Engineering of Brassica napus Seeds for Oil Quality and Yield with Toxicological and Motor-Technical Aspects of Biodiesel in Mind.*

R Jördens: I represent the German Ministry of Food, Agriculture and Forestry. I wish to outline the German Government's view on these matters. First, a quick overview of our policy on renewable resources (Table 1).

Table 1. Government policy for renewable resources.

Aim:	Protection of fossil resources; environment help farmers; rural areas → stimulate demand for agriculture raw material to be transformed into high value products
Instruments:	improve framework conditions regulations, reform of CAP, taxes,...
	R&D: - starch - wood/lignocell - sugar - proteins/others
	(55 Mio DM p.a.) - oils - energy - fibres = solid biomass = liquid fuels
Results:	steady and stable increase 520.000 ha (1996) out of 12 Mio ha = ~4 %

Our aim is to protect both scarce fossil resources and the environment, especially the climate and, of course, as the Ministry of Agriculture, to help farmers and rural areas. We thus try to stimulate the demand for agricultural raw materials to be transformed into high-value products. This is very important and is

our specific goal. What instruments are available? We are trying to improve structural conditions, for instance market regulations for starch and sugar and, of specific importance, to reform Common Agricultural Policy, which has brought the prices of agricultural products closer to the world market level. This of course helps renewable resources. A further instrument is taxation. Very important, and relevant here, is our support for research and development. Our programme is of a magnitude of about 55 Mio. DM p.a. and is organised according to different production lines - starch, sugar, oils, fibres, lignocelluloses, proteins and, importantly, energy. This consists of two parts: solid biomass for burning and production of electricity and heat, and—our theme here—liquid fuels. What has been achieved? We note a stable and steady increase in utilisation of renewable resources in all these production lines and, consequently, an increasing acreage. Last year it was 520,000 ha, about 4 % of the total 12 Mio. ha arable land in Germany. One part of this is vegetable oil, rapeseed oil for liquid fuel production.

What is the importance of Biodiesel in Germany at present?

Table 2. Importance of cost and RME in Germany (1996)

Diesel consumption	26 Mio t
RME	50.000 t (~0.2 %)
15 % RME	4 Mio t
	4 Mio ha
arable land	12 Mio ha
Production cost RME on set aside areas	
(27 DM/dt rapeseed)	~1.20 DM/l
Production cost on regular land	~2.30 DM/l
Diesel price (without tax)	~0.30 DM/l

Overall diesel fuel consumption in Germany is about 26 Mio. t/year. RME consumption is 50,000 t, i.e. 0.2 %. It has been suggested that RME could contribute 15 % of diesel consumption. This means a production of 4 Mio. t and an area of 4 Mio. ha. Since 12 Mio. ha are available, these figures show this percentage is unrealistic on present basis. What are the reasons for this? Very simply, RME has to compete on the market with a very cheap product, diesel fuel, which costs 30 Pf/l without tax. What are the production costs of rapeseed RME? RME can be produced only on set-aside areas, areas that can be used only for non-food crops. There is a limited outlet for the product, which means a very low price for the farmer for the rapeseed. This brings us to the production costs of about 1.2 DM/l. Competition with diesel is only possible because mineral oil tax has to be added to diesel, and not to RME. This is the reason for the similar prices at present. If RME were to be produced on regular land there would be no chance of competing with the diesel price because here the hectare prime which is accorded to the farmer producing rapeseed oil must be deducted and the production costs rise to 2.3 DM/l.

What is happening on set-aside land, and what is done with the rapeseed oil coming from the set-aside?

Table 3. Utilisation of plant oils and fats as renewable resources (tons/hectares).

from harvest in year	oleochemicals (t/ha)	lubricants (t/ha)	Biodiesel (t/ha)	export (t/ha)	total (t/ha)
1993	60.000	15.000	5.000	40.000	120.000
1994	70.000	25.000	25.000	55.000	175.000
1995	100.000	30.000	30.000	80.000	240.000
1996	110.000	35.000	50.000	60.000	255.000

There are four forms of utilisation: oleochemistry, currently using 110,000 t/ha. The lubricant industry currently produces and utilises 35,000 t and Biodiesel 50,000 t. The most important is—and here we have the field with the highest value obtainable—oleochemistry. There are possibilities here as well.

What research priorities have been set?

Table 4: Government programme, R&D renewable resources (1996-2000)

Energy from biomass (vegetable oils)

Status:
- high energy density
- rape in Germany most productive; (sunflower)
- different technical approaches

Research needs:
- technical approaches more or less optimised - no need for funding R&D
- some (limited) effort still useful on environmental effects
- quality questions; quality control; standardisation

Research priorities have been laid down in a publicly available programme. There are brief remarks on the status of biofuels, energy from biomass and the status of vegetable oils. Vegetable oils have a high energy density and are easy to extract. In Germany rapeseed is the most productive cultivar and, perhaps in some other areas of western Europe, sunflower. Different technical approaches have been developed since 1973, and the research needs are as follows: the technical approaches are more or less optimised. There is no need for further funding of R&D. This is stated in the Government's programme. Some limited effort in the direction of environmental effects of using biofuels and concerning quality tests, quality control and standardisation is still necessary.

P Walzer: Does the higher yield potential necessitate a higher fertiliser supply and, thus, enhanced leaching of nutrients?

G Kley: In the last decade, nutrient efficiency of winter oilseed rape varieties has been improved, so that in consequence the risk of leaching has been diminished. Three factors are important here:

1. The management of nitrogen fertilisation has been improved progressively; required amounts have been determined from analytical data from soil and weather conditions and fertiliser amount adjusted to yield potentials, thus economising on fertiliser as well.
2. Progress in analysing soils and adjusting fertilising level to yield potential has led to a general reduction in the amount of fertiliser required per ha for rapeseed production in Germany:

	Past (1980)	improved varieties	Currently applied (1995)	within a few years
N-units per ha	220		180	
yield kg/ha	2.700	3.000	3.500	4.000
N-unit/kg yield	0.081	0.073	0.051	0.045
Percent	100	90	63	56

3. Selection of improved germplasm usually is conducted under constant fertiliser regime. This automatically results in varieties with more efficient nutrient use.

P Walzer: Dr Kley, this is correct, but possibly difficult to communicate. The question is: what would the consequences be of not using a set-aside field at all, or using it for fuel production? If positive aspects can be deduced from this comparison, this would be something which we can communicate.

G Kley: It is correct to ask the question: what elements or influencing factors are to be compared. A field without any biomass production has more nitrogen leaching than a field covered with a crop.

Roundtable Discussion

J Schell: In the following roundtable discussion, I would ask the discussants to give a statement and to identify themselves and their interests.

R Töpfer: I am Reinhard Töpfer. I was a co-worker of Jeff Schell for roundabout a decade and am still interested in rapeseed and Biodiesel. There are still research activities required and yield is one of the top priorities for improving the long-term possibilities of Biodiesel. There are also trends towards improving the biofuel itself by changing the composition of the fatty acids. However, definition of the types of fatty acids required is hindered by a lack of information. There is need to take a given motor and test this motor for certain types of synthetically made methyl ester combinations. This could be a starting point for obtaining more and clear-cut information for plant breeding with respect to changing the fatty acid composition. To summarise: the first point is increasing yield, the second close interaction with engineers developing and improving motor characteristics to find a desired and optimised biofuel based on methyl esters.

P Walzer: I am Peter Walzer. My interests are in engine development and I work with FEV Engine Technology in Aachen. Given the very small amount of rapeseed oil currently available in Germany, first priority is that the fuels coming from biomass should fulfil the DIN-norm for plant methyl ester. Secondly, a far higher yield of rapeseed oil biomass was demanded, an increase of at least 100 %. On the issue of specific requirements for rapeseed oil-derived fuels I agree with Mr Töpfer that our knowledge is not currently adequate, even on the engine side, with respect to the components of the plant oil that should be changed. Some requirements have been mentioned, for instance less polyunsaturated fatty acids. I also agree that synthetic oils are necessary, and that these should be investigated in a very modern, high-pressure direct-injection, exhaust gas-recirculated diesel engine—this is really a new world—to establish the right directions for change. A new aspect is just how broad the spectrum is in terms of modified fatty acid compositions in plant oils. This might open a lot of possibilities and this should be investigated.

J Schell: I am Jeff Schell, one of the organisers. I am very pleased that, for the first time, parties with vested interests have declared and discussed these with each other. One thing is clear, nothing will be possible if it does not make economic sense. Very important is that there are three ways of approaching the problem, modify the engines, modify the additives, the "whatever" the engines receive, and modify the plants. The notion was raised that *dedicated engines* are currently not economically feasible and this we have to accept. But *dedicated*

plants are indeed possible. This is a pragmatic reason for going in that direction. This is not to say that the other possibilities should be ignored.

O Syassen: My name is Syassen, I'm a consultant for diesel engines, especially with Biodiesel. I would like to make three short remarks: firstly about the percentage of NO_x with gas oil and PME in comparison. The slightly (usually about 8 %) higher NO_x emissions with PME are not only a consequence of the different chemical structure of PME: This was not quite clear. The main reason is physical, namely the high compression module of the PME. That means that PME is hydraulically "harder" than diesel fuel or gas oil and, because the engines have more-or-less weaker hydraulic injection systems, the injection delay between start of the working of the injection pump and the start of the fuel injection into the cylinder will be different with these different fuels. If nothing is changed in the engine, the injection and - including the better ignition quality - the start of ignition will be earlier with PME than with gas oil. In general, for PME and diesel fuel, if the ignition starts earlier combustion pressure and the maximum temperature are higher and hence more NO_x will result. In other words, the higher NO_x emission is mainly a physical problem of the engine rather than a chemical problem of the fuel (oxygen content). Importantly, for the same ignition start, NO_x emissions from PME are about the same, or even lower, than with diesel fuel. The second point is that the absolutely most important factor for the future development with Biodiesel is the quantity of biofuel that will be available in the next decades. The figures have differed from 1–2 % of total diesel consumption up to about 12 %. In between there lies a world of useful strategy for the next years because this factor is so decisive that more realistic figures are absolutely essential. There are many consequences and many influences, for example, what is the situation with the Blair House compromise? We need to know in the next years whether the possibilities are going to be about 1 % or about 10 %. The final point is that it must be made absolutely clear that all current emission figures so far have been derived from diesel engines optimised for fossil diesel. These are, as special test runs with small engines have shown, quite different to engines optimised for Biodiesel. Whether or not such engines are suitable for serial production is a question of economics, and the numbers are so low that at the moment there is no useful chance. Quite another question is whether a test engine should be developed, which is probably not a big problem, and would give more realistic results for the Biodiesel strategy and for developmental work on both fuel and engines.

W Körbitz: With respect to potential and the view of the German Ministry of Agriculture: I have different figures, and when I said our prognosis was ambitious, on the basis the European Commission's figures, my view after hearing the plant breeders is that it is not ambitious, but rather realistic. On the basis of 12 Mio. ha in Germany and 26 Mio t diesel, allowing 10 % for Biodiesel—the European Commission assumes about 12 % for total biofuels including bioethanol so one can assume 10 % for Biodiesel—this is 2.6 Mio t Biodiesel. The information has been that oil yield per hectare can be doubled, meaning 1.3 Mio. ha. Deducting

1 % for recycled waste oil gives less than 10 % of the total German acreage. Given set-aside percentages of 10–15 % this sounds realistic.

Concerning potential research, it is fortunate that Biodiesel from double-low rapeseed oil is an ideal product. We are seeking to broaden this basis to lower cost and, if possible, improve quality and flexibility. One answer is high oleic, ideally 100 % oleic fatty acid methyl ester. Although 100 % cannot be achieved, if the high oleic also has a low linolenic profile, this may serve the interests of the engine industry much better. This is the reason why a discussion of a new DIN standard need not be opened, but research into the iodine number is necessary, as the information is not sufficiently precise. This is not to question the DIN, we all support the high-quality philosophy to reach a standard, but if research addresses the limitation of triple-unsaturated, linolenic, fatty acid, a lower iodine number may result. The third point is the consequence of moving towards a lower boiling point closer to diesel, which can be reached probably with shorter chains? There is very little experience with short- or, long-chain saturated, mono-unsaturated fatty acids and their blends. This is certainly a very interesting field because it will lead to broadening the raw material basis and hopefully to lower cost.

Concerning engines, if we look not at dedicated engines, but dedicated use of engines in certain market niches, like a boat running on 100 % Biodiesel permanently, it might be feasible to consider ignition timing in this instance, and not complicate electronic systems generally to reduce NO_x down. The engine industry appears not to be worried about cetane. If this is to be increased, which is possible, this must be communicated to the plant breeders. The last point is toxicology. There appears to be no clear information on emission toxicity.

G Oberdörster: I am Günter Oberdörster, head of the Division of Respiratory Biology and Toxicology of the Department of Environmental Medicine at the University of Rochester. Using literature data, we have evaluated petroleum diesel exhaust with respect to toxicity and risk estimation. In general, it would be most desirable if there were no carcinogenic, mutagenic or irritancy potential related to inhalation of diesel exhaust i.e., no particles, PAH or irritating substances like aldehydes and acrolein etc- This of course is a very utopic view since any high-temperature combustion will generate these substances. There are numerous results with respect to petroleum diesel that can be compared with the few data from Biodiesel. Most studies show that there is a lower particle and PAH content in Biodiesel exhaust, although NO_x, specifically NO_2, is increased, as are potentially irritating aldehydes. The studies that compared the effects of these compounds with respect to mutagenic potential using very simple systems like the Ames assay on *Salmonella* or other simple mammalian cellular assays seem to show a lower mutagenic potency for Biodiesel exhaust than for petroleum diesel exhaust or exhaust extract. Using the petroleum diesel studies as a benchmark, it may be concluded that Biodiesel used in combustion engines may be better and may have an advantage with respect to carcinogenicity and mutagenicity.

However, although this prediction is probably sound, one problem with extrapolation to in vivo is that it is difficult to predict the overall long-term effect of inhalation in a mammalian organism just from particle measurements and knowledge of particle size distribution in Biodiesel exhaust, and whether this will

be the same as for petroleum diesel exhaust. Thus toxicological studies in mammals are needed to find out more about long-term in vivo effects. For example, a short-term study on Biodiesel exhaust could be designed using inhalation and laboratory rodents and the results compared with petroleum-based diesel exhaust. Only when there are at least preliminary or pilot study-type data from such a comparison can predictions or statements be made on the potential of Biodiesel exhaust with respect to inflammatory effects and mechanisms, fibrosis, and potentially cancer and also mutagenicity. In the absence of such data no well-founded suggestions can be made to the plant breeders as to what may need to be altered in Biodiesel fuel in terms of specific constituents to reduce toxicity.

The other problem is that development in engine design is ongoing and is aimed at changing the combustion process. This will of course change the exhaust components. Moreover, extensive research into the breeding of biofuel plants is going on, and transgenic technology being introduced, to improve both the plant and the fuel. Until these two areas of research have produced at least preliminary results, resulting in a standardised or reference fuel and a more-or-less been standardised engine, there may not be much point in carrying out toxicological testing of the kind just mentioned. The conclusion from our session on toxicology was that the only suggestion for the plant breeders at this point is to try and to increase the oil yield per hectare as much as possible.

N Martini: I have been at the Max Planck-Institute for Plant Breeding in Cologne as a collaborator of Jeff Schell for more than 10 years. I am also one of the organisers. My statement is mainly twofold. There is a portfolio of different rapeseed oils which are available for possible use as RME. More research is needed into the production of a particular unique fatty acid in the oil, for instance, the high oleic acid rapeseed methyl ester which would be favourable for its low iodine number, because of its reduced polyunsaturated fatty acid content. The other point is the economic aspect, the increase in yield per hectare. There, biotechnology can contribute with an array of research activities. One particular contribution is the increase in oil content within the seed, i.e. the increase of the ratio oil to protein or starch, leading to a decrease in starch and other storage compounds. There are cases in the plant kingdom where approximately 100 % more oil is stored than in rapeseed. If the genetic determination of this phenomenon is understood and the responsible genes identified, genetic engineering will achieve this also in rapeseed.

J Schell: Are there questions?

G Röbbelen: I must contradict the Chairman of the Panel. If I understand correctly, a dedicated engine, i.e. an engine specifically made just for Biodiesel, is no commercial possibility. If this is so, then the conclusion that a dedicated plant can be produced, is not possible either. A rapeseed plant tailored just for fuel is not economically feasible. "Optimised" is the word I would use in both cases: produce an optimised test engine and an optimised plant containing as much oleic as possible, more than 90 %.

J Schell: That "optimised" is a better term than "dedicated" is not disputed. I just wanted to use terms that had been used here before.

J McDonald: Dr Syassen, I must disagree with some of your comments on to oxides of nitrogen emissions. They ignore the effects of local equivalence ratio due to the increased oxygen content in the fuel and the effects that has on oxides of nitrogen emissions. There is considerable data in the literature on petroleum diesel fuels with oxygen additives less than that of the oxygen content of soy methyl esters that shows a small but still statistically significant increase in oxides of nitrogen emissions. Consequently, the US Environmental Protection Agency has decided to limit the oxygen content of standard diesel fuels in the USA to 1 %. They believe that the oxygen content alone is one variable that affects oxides of nitrogen emissions. In addition, my research just using an inline pump and nozzle injection system on a Caterpillar 3300 series engine showed not much more than a half-degree crank angle difference at 2200 rpm, the maximum operating speed of the engine, between the start of injection comparing soy methyl ester with low-sulphur petroleum diesel fuel, even though the physical characteristics were quite different. Finally, your commentary also ignores differences in the dynamics of the injection system, because the newer unit injection systems probably will be affected even less than our inline pump and nozzle injection system.

O Syassen: The injection delay was only half a degree? This already will contribute to NO_x.

J McDonald: Yes, but the typical retard used in bus engine testing in the USA with unit-injected engines, which will probably have even less difference. A 20 % blend of soy methyl ester means only about a 2 % oxygen content. With the 6V92 Detroit Diesel engines, a 2–3° retard is typically used to equal the oxides of nitrogen emissions of petroleum diesel fuel, and I believe that the dynamics of the pump system are significantly less than this retard.

J Schell: The discussion is becoming too technical. Could you say simply in what respect you disagree with Dr Syassen's conclusion?

J McDonald: It's the oxygen content of the fuel and not the hydraulics.

J Schell: You would conclude that both fuels and engines can be manipulated?

J McDonald: That oxides of nitrogen emissions are often a function of the oxygen content of the fuel. The engine can be manipulated to bring the oxides of nitrogen emissions down, that also affects particulate matter emissions.

G Röbbelen: Fuel oxygen content has been discussed repeatedly, but no figures have been mentioned. Is it not possible to say what the percentage of oxygen is that can be used in our plant oils for transesterification?

P Walzer: This is 10–11 % with today's RME or PME standard. This is much more than in diesel fuel. But the question is, what might be achieved? This I would like to pass on to the Max Planck Institute.

J Schell: The question we put to you is rather what is going to be optimal, not just what can be achieved. One of the consequences of our discussions is that the limit with which to begin is unknown. Before beginning, we need to know what is optimal. It must be realised that nothing can be achieved that does not already exist in nature. So that is of course the limitation. But anything that exists in nature and that could be brought to bear, as long as we know exactly what is optimal, we could attempt to put into rapeseed.

W Körbitz: I showed a curve from Professor Sams relating soot reduction to fuel oxygen content. The curve rose quite steeply up to 10 % but flattened out, so that at 15 % there was not much more improvement in soot reduction. For the 10 % soot reduction was 60 %. Oxygen content must be examined, and compared with all other parameters. There is no indication that it should be increased further, which would be the case if shorter chains were used, but reducing oxygen content might be an option.

J McDonald: That raises another question. It all depends on what is intended for the RME, and other Biodiesel for that matter. If it is to be used it as an additive to diesel fuel, then as high a cetane number or oxygen content as possible would be desirable. For good economics, no more than a 30–40 % blend in petroleum diesel fuel would be needed to achieve an oxygen content that would lower soot, and a high cetane number might improve the oxides of nitrogen emissions. It was questioned whether the cetane number in fuel should be maximised. Most on-highway diesel engines today have fairly short ignition delays and it may be asked whether a cetane number much more than 50 is needed. I think this is the current EU standard. In the United States it is 40.

F Adlkofer: I have a question for Professor Oberdörster. One of our aims was to establish out whether Biodiesel would be preferable to normal diesel on the basis of available toxicological findings. If so, this would certainly enhance the marketing of Biodiesel. We have heard that this might be so, but that we are far from being able to claim that it is true. Would you agree with this?

G Oberdörster: We should probably not say "far" away. The prediction, that in all likelihood Biodiesel exhaust has better toxicological properties than petroleum diesel exhaust, is fairly sound. All the presently available data point in that direction, but toxicological studies in mammalian species are necessary. We are as far away as the result of an animal inhalation study with Biodiesel exhaust. This can be performed as soon as a reference or standard fuel and a "reference" engine have been identified.

J Schell: Mr Mauderly, would you like to add to this?

J Mauderly: I agree that we are not far away. There are data from different fuels, different engines and different studies. It would be easy to say that this does not constitute a coherent body of information, that the studies must be repeated with a specific fuel or a specific engine. In fact, Biodiesel technology will change, and evolve, just as petroleum diesel technology has. Engines and fuels will change and will continue to do so. We will thus never reach the ideal situation of being able to do the last convincing study. The information that we have suggests clearly that emissions from engines burning Biodiesel will not be worse than those from petroleum diesel engines. From a toxicological viewpoint they will probably be somewhat better. There are certain studies that still necessary to give us more confidence that the entire spectrum of effects of interest has been covered. Some of these studies simply have not been done with any combination of fuel or engines. These are not difficult studies, nor are they protracted. These are studies to see how the intact organism responds to the complete inhaled mixture of exhaust, the irritant effects such as coughing or asthma or complications with lung conditions. Even with today's engine technology and fuels this would not be difficult. The toxicologists must watch the development of the technology to identify questions arising as the technology changes. At present, toxicologists and health professionals would be satisfied with a small amount of additional information that there are no critical problems in sight.

H Klingenberg: I return to my argument that the impact on humans and traffic will be very low for the current population with diesel cars. A 20 % contribution by Biodiesel—a very optimistic value—will result in a negligible change in risk.

G Oberdörster: This is correct. However, the general trend is to reduce particulate and PAH emission as much as possible. So it would be interesting if Biodiesel were no worse than fossil diesel. This appears not to be the case, but we need to know something about it. Thus whilst Biodiesel may not make a tremendous improvement if it contributes 20 % to the overall emissions, to be firm in our statements we still need to know something about its toxicity after inhalation compared to petroleum diesel

J Schell: Professor Klingenberg, have you reached a different conclusion?

H Klingenberg: Yes. In the end, one has to be able to calculate the risk to allow a judgement on whether the impact on humans is reasonable or improved.

G Oberdörster: Certainly. Precisely for that reason we need knowledge on the inhalation toxicity of Biodiesel exhaust, particularly its effects on the more susceptible part of the population, for instance asthmatics.

H Heinrich: I would like to stress another point. Only one person will decide whether PME is a success or not, the customer. If he is satisfied with the economics and with the behaviour and the lifetime of his car, including the properties of the fuel, then PME will make its way. All activities of the plant breeders to optimise the crop yield and to optimise the economics are thus

welcome. In any case a high-quality product is necessary. There is no sense in further discussing DIN V 51606, because that fuel and the cars are on the market. What is necessary now is safety for the customer. That means we need the final standard as soon as possible. With this norm we would be well on the way to bring PME to the customer and onto the market.

W Körbitz: I support the high-quality philosophy fully and always have. But the diesel norm has changed, norms anyway are subject to any change: the better one is the enemy of the good one. I have two objectives: to fight for the final DIN so as to obtain the confidence of the customer and to have an open discussion with the engine industry to consider a better one, which could follow in the next years.

H Heinrich: I agree completely. However, beginning a scientific struggle over the value of this norm will loose much time. It must be in place as soon as possible, preferably already in 1997. I doubt whether the representatives of the automotive industry on the norming panel will vote for a change e.g. in the iodine number.

G Kley : May I contribute to the question of customer orientation. An amusing aspect was the report of experience from the gas stations distributing Biodiesel. Biodiesel was most attractive to the elderly and the young, but not to the middle-aged. There are thus two options for customer orientation: focus on the elderly, who will disappear one day, or the young, who will form the future sales opportunities.

H Heinrich: I agree, but even the young will lose interest in this fuel if their engines fail, if there is trouble with the fuel, with the engines or even with the economics. We need the same criteria to satisfy the customer.

J Schell: I'm sorry to interrupt this lively discussion. As an old person, I think I must say that we are looking forward to changes even if it brings advantage only to part of the consumer population. I think old people are wise enough for that. I would like to thank all who have participated.

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